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ALLEN'S COMMERCIAL ORGANIC ANALYSIS

FIFTH EDITION, REWRITTEN, REVISED, RESET
COMPLETE IN TEN VOLUMES EACH VOLUME SOLD SEPARATELY

The organic chemicals and products employed in the arts, manufactures, commerce, medicine, science, etc. It treats upon the properties, modes of analysis, proximate analytical examination; methods for detection and estimation of impurities, adulterations, products of decomposition, etc.

CONTENTS OF VOLUME I

- Introduction.** By W. A. DAVIS, B. Sc., A. C. G. I., Rock Ferry, Cheshire, Eng. Preliminary Examination; Specific Gravity; Changes in Physical State; Optical Properties; Spectrometers and Spectrographs; Polarimeters; Arrangements for Maintaining a Known Constant Temperature; Ultimate Analysis; Moisture, Crude Fibre and Ash; Action of Solvents.
- Alcohols.** By L. M. BURGHART, M. A., Baltimore, Md. Methyl Alcohol; Wood Naptha; Crude Wood Spirit; Acetone; Ethyl Alcohol; Higher Aliphatic Alcohols.
- Malt and Malt Liquors.** By JULIAN L. BAKER, F. I. C., Staines, Eng. Malt; Malt Worts; Roasted Barley and Malt; Brown and Crystal Malts; Malt Substitutes; Grits and Raw Grain; Malt Extract; Caramel; Invert Sugar; Starch Sugars; Preparation of Materials; Beer and Ale.
- Wines and Potable Spirits.** By LEWIS EYNON B. Sc., F. I. C., London. Wines; Significance of Results of Wine Analysis; Cider; Potable Spirits.
- Yeast.** By EMIL SCHLICHTING, PH. D., New York. Yeast; Culture Yeast; Pure Culture of Yeast and Its Application in Practice; Physical Examination of Yeast.
- Neutral Alcohol Derivatives.** By HENRY LEFFMANN, M. D., PH. D., Philadelphia. Ether; Aldehydes; Method of Determining Chloroform in Medicinal Preparations.
- Sugars.** By LEWIS EYNON, B. Sc., F. I. C., London. Classification; Methods of Analysis Depending on Specific Gravity or Solution Density; Methods of Analysis Depending on Optical Activity; Method of Analysis Depending on Refractive Index; Methods of Analysis Depending on Reducing Power; Method of Analysis Depending on Oxidation with Iodine; Methods of Analysis Depending on Fermentation; Cane Sugar, Analysis and Valuation of Cane and Beet Sugar Products; Sucrose in Beetroot; Maltose; Lactose; Monosaccharides; Honey; Maple Products; Urine Analysis; Pentoses.
- Starch and Its Isomerides.** By T. H. POPE, B. Sc., F. I. C., Wallasey, Cheshire, Eng. Starch; Estimation of Starch; Dextrine, Amylin; Cellulose; Gums;

Proximate Analysis of Plants; Cereals; Wheat, Flour; Bread; Macaroni; Vermicelli, Spaghetti, Noodles, etc., Biscuits and Milk Flour; Other Cereals.

Paper and Pulp Testing. By E. SUTERMEISTER, S. B., Westbrook, Me. Paper; Physical Tests; Chemical Tests, Wood Pulp.

Aliphatic Acids. By HUGO SCHLATTER, M. S., Wilmington, Del. General Reactions; Acetic Acid; Vinegar; Homologues of Acetic Acid; Malic Acid; Tartaric Acid; Tartrates; Citric Acid; Citrates; Lactic Acids.

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CONTENTS OF VOLUME II

Fixed Oils, Fats and Waxes. By C. AINSWORTH MITCHELL, M. A., F. I. C., London. General Properties and Analytical Methods; Extraction and Purification of Fixed Oils and Fats; Constitution and Chemical Properties of Fats, Oils and Waxes; Hydrogenerated Oils; Colour Tests of Oils; Physical Properties of Oils; Classification of Fats, Oils and Waxes; Identification of Fats and Fixed Oils.

Special Characters and Modes of Examining Fats, Oils and Waxes. By C. AINSWORTH MITCHELL, M. A., F. I. C., London. Olive Oil Group; Rape Oil Group; Cottonseed Oil Group; Linseed Oil Group; Castor Oil Group; Cacao Butter Group; Lard Oil Group; Tallow and Butter Group; Whale Oil Group; Sperm Oil Group; Beeswax Group.

Butter Fat. By CECIL REVIS and E. R. BOLTON, London. General; Qualitative Tests; Butter; Estimations; Preservatives; Margarine; Hardened Fats; Vitamines; Ghee.

Lard. By GEORGE A. REITZ, B. Sc., Ph. C., Philadelphia.

Linseed Oil. By GLENN H. PICKARD, Minneapolis. General; Iodine; Hexabromide Test; Other Tests and Methods, Oxygen Absorption; Polymerised Oil; Refining; Air Treated Oils; Boiled Oil; Effect of Storage; Detection of Adulterants.

Higher Fatty Acids. Revised by H. E. COX, M. Sc., Ph. D., F. I. C., Newport. Characteristics; General Properties; Separation of Mixed Fatty Acids; Palmitic Acid; Stearic Acid; Oleic Acid; Sebacic Acid; Elaidic Acid; Sulfopholeic Acid.

Soaps. By ELBERT C. LATHROP, A. B., Ph. D., Philadelphia. General; Detergent Action; Raw Materials; Alkalies and Fillers Varieties; A. C. S. Methods; Separation of Unsaponified Matters; Phenols; Examination for Special Constituents; Soap Powders, etc.; Interpretation of Analyses; Specifications.

Glycerin. By J. W. LAWRIE, Ph. D., Wilmington. Uses for Glycerin; Pharmacopocia Test; Qualitative Test; Estimation; Specific Gravity; Refractive Index; Boiling Points; Analysis of Crude Glycerin; Analysis of Refined Glycerin; Pure Glycerin; Moisture in Glycerin; Foos; Polyglycerins; Fermentation Glycerin; Glycerin in Wines; Physical Constants.

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Sterol Alcohols. By JOHN ADDYMAN GARDNER, M. A., F. I. C., London. Cholesterol; Vegetable Sterols; Sources of Sterols; Phytosterol; Amorphous Sterols; Extraction of Sterols; Colorimetric Methods.

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Bitumens. By SAMUEL P. SADTLER, PH. D., L. L. D., Philadelphia. Natural Gas; Petroleum; Distillation of Petroleum; Naphtha; Kerosene; Gas Oil; Lubricating Oils; Lubricating Greases; Petrolatum; Paraffin; Asphalt; Asphalt Fluxes; Asphalt Pavings; Roofing Papers; Bibliography.

Napthalene and Its Derivatives. By W. A. DAVIS, B. SC., A. C. G. I., Rock Ferry. Napthalene; Napthalene Oils; Naphthols; Naphthol Ethers; Naphthol Sulphonic Acids; Bibliography.

Anthracene and Its Associates. By JOHN H. SACHS, PH. D., Wilmington. Anthracene; Anthraquinone; Phenanthrene; Carbazol; Compounds with Picric Acid; Valuation of Anthracene; Bibliography.

Phenols. By J. BENNETT HILL, PH. D., Philadelphia. Monohydric Phenols; Phenol; Cresols; Xylenols; Commercial Carbolic Acids; Dip and Flotation Oils; Creosote; Cresylic Acid Disinfectants; Dihydric Phenols; Guaiacol; Wood Creosote; Trihydric Phenols; Bibliography.

Aromatic Acids. By EDWARD HORTON, B. SC., London. Sulphonated Phenols; Naphthol Sulphonic Acids; Benzoic Acid; Metallic Benzoates; Benzoic Esters; Benzoic Aldehyde; Oil of Bitter Almonds; Saccharin; Cinnamic Acids; Cinnamic Esters; Cinnamic Aldehydes; Oil of Cinnamon; Coumarin; Gum Benzoin; Peruvian Balsam; Tolu Balsam; Liquid Storax; Salicylic Acid; Metallic and Alkaloidal Salicylates; Salicylic Esters; Derivatives of Salicylic Acid; Homologues of Salicylic Acid; Hydroxy-toluic Acids; Dihydroxy-benzoic Acids; Vanillin; Bibliography.

Gallic Acid and Its Allies. By W. P. DREAPER, O. B. E., F. I. C., London. Gallic Acid; Esters and Derivatives of Gallic Acid; Pyrogallol; Bibliography.

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Picrates; Dinitrophenol; Nitrotoluenes; Trotyl or T. N. T.; Nitrochloro benzenes; Nitronapthalenes; Tetranitromethylaniline (Tetryl); Mercury Fulminate; Gelatinizers and Stabilizers; Diphenylamine; Moisture in Explosives; Analysis of Complex Explosives; Fireworks; Detonators; Abel Heat Test; U. S. Directions for Abel Test; Significance of Heat Tests; Fume Tests; Quantitative Tests; Chemical Methods; Bibliography.

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CONTENTS OF VOL. IV

Essential Oils, Special Characters. By E. K. NELSON and G. A. RUSSELL.

Cymbopogon oils; Indian Grass Oils; Almond Oil; Angelica Oil; Bay Oil; Oil of Bergamot; Buchu; Calamus; Cajuput; Caraway; Cardomon; Cassia; Cinnamon; Camphor; Cedar-wood; Cedar-leaf; Chamomile; Champaca; Chenopodium; Clove; Coriander; Cubeb; Cumin; Cypress Leaf; Dill; Eucalyptus; Fennel; Ginger; Geranium; Hyssop; Hop; Juniper; Lavender; Spike-Lavender; Lemon; Pinene in Lemon Oil; Lime; Linaloe; Nutmeg; Mace; Orange; Petitgrain; Orange-Flower; Parsley, Peppermint; Spearmint; Pennyroyal; Pine-Needle; Rose; Otto of Roses; Attar of Roses; Rosemary; Rue; Sandalwood; Sassafras; Sweet Basil; Snake Root; Thyme; Turpentine; Wormseed; Wormwood; Wintergreen; Ylang Ylang; Canaga; Terpeneless Essential Oils; Tables of Characters and Constituents of Essential Oils.

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The Synthetic Dyestuffs. By A. W. JOYCE, PH. D., New York. Classification of the Synthetic Dyestuffs; The Nitroso Dyestuffs; The Nitro Dyestuffs; The Azo Dyestuffs; The Basic Azo Dyes; Acid Monazo Dyes; Monazo Dyes from Ortho-aminophenols; The Ice Colours; Pyrazoline Dyes; Acid Disazo Dyes; Substantive or Direct Cotton Dyes; Direct Cotton Dyes Derived from Diamines of the Benzene and Naphthalene Series; Direct Cotton Dyes Derived from J-acid and Its Derivatives; Dyes from Thiazole Bases; The Stilbene Dyes; Ketonimines; The Carbonium Colouring Matters; The Arylmethane Dyes; Diamino Derivatives; Triamino Derivatives; Amino-oxy-derivatives; Oxy-derivatives; Diphenyl-naphthyl Methane Dyes; Xanthene Dyes; The Amino Derivatives; The Hydroxy Derivatives; Amino-hydroxy Derivatives; Acridine Dyes; Quinoline Dyes; Thiazole Dyes; Indophenols and Indamines; Oxazine Dyes; Thiazine Dyes; Azine Dyes; Sulphur Dyes; Anthraquinone Dyes; Mordant-dyeing Anthraquinone Dyes; Anthraquinone Acid Dyes; Vat Dyes; Indigo and Indigoid Vat Dyes; Benzoquinone Vat Dyes; Identification of Azo Dyes; Reduction Products of Azo Dyes and the Dyes from Which They are Obtained; Physical and Chemical Properties of the Reduction Products from Azo Dyes.

Analysis of Colouring Matters. By HANS EDWARD FIERZ-DAVID, D. SC., Zurich, Switzerland and V. E. YARSLEY, D. SC., M. SC., A. I. C., General; Colour Standards and Colour Comparison; Aniline Lakes; Spectroscopic Investigation; Qualitative Investigation of Dyestuffs in Substance; Qualitative Investigation of Dyestuffs on Animal Fibre; Triphenylmethane Dyestuffs; General Procedure; Treatment of Mixtures; Qualitative Investigation of Dyestuffs on Vegetable Fibres; Preliminary Investigations; Chemical Reactions of the More Important Classes of Dyestuffs; Quantitative Analysis of Dyestuffs; Relative Methods; Absolute Methods; Titration with Hydrosulphite; Bibliography.

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General Section on Alkaloids. By T. M. SHARPE, M. SC. TECHN. A. I. C. London. Alkaloids of Alstonia Bark; Alkaloids of Areca or Betel Nut; Alkaloid of Barley Germs; Alkaloids of the Common Broom; Alkaloids of the Calabar Bean; Alkaloids of Delphinium; Alkaloids of Ephedra Spp.; Alkaloids of Ergot; Toxicological Investigation of Ergot; Alkaloids of Hemlock; Poisoning by Coniine and Hemlock; Assay of Hemlock and its Preparations; Alkaloids of Holarrhena Spp.; Alkaloids of Ipecacuanha; Alkaloids of Jaborandi; Alkaloids of Yellow Jasmine; Alkaloid of Laburnum and Furze; Alkaloids of Lobelia Inflata; Alkaloids of Lupuis; Alkaloids of Meadow Saffron (Colchicine); Assay of Colchicum; Toxicology of Colchicum; Alkaloids of Mescal Buttons; Alkaloid of Papaya; Alkaloids of Pegamum Harmala; Alkaloids of Yagé and Caapi; Alkaloids of Pepper; Analysis of Pepper; Alkaloids of Pomegranate; Alkaloids of the Potato, etc.; Alkaloids of Labadilla; Alkaloids of the Hellebores (Veratrum); Alkaloid of Yew; Alkaloids of Yolumba Bark; Alkaloids of Aspidosperma Quebracho Blanco; Bibliography.

Aconite Alkaloids. By FRANCIS H. CARR, C. B. E., F. I. C., London. *Species of Aconite Plants; Constitution and Characters of the Aconite Bases; Aconitine; Salts of Aconitine; Chemical Reactions of Aconitine; Derivatives of Aconitine; Benzaconine; Aconine; Pyraconitine; Pyraconine; Amorphous Alkaloids of A. Napellus; Japaconitine; Indaconitine; Pseudo-aconitine; Bihhaconitine; Jesaconitine; Lycaconitine and Myoctonine; Lycoctonine; Myoctonine; Lapaconitine, Leptentrionaline and Cynoctine; Atisine; Assay of Aconite Root and its Preparations; Toxicology of Aconite; Toxicological Detection of Aconite; Pharmacology of Aconite.*

Berberine and Its Associates. By E. HORTON, B. Sc. Berberine; Constitution of Berberine; Reactions and Detection of Berberine; Estimation of Berberine; Volumetric Methods; Gravimetric Method; Salts of Berberine; Oxyacanthine; Berbamine; Hydrastine; Estimation of Hydrastine; Hydrastis Rhizome; Hydrastinine; Salts of Hydrastinine; Canadine; Calumbin.

Caffeine, Tea and Coffee. By J. J. FOX, D. Sc., F. I. C. and P. J. SAGEMAN, F. I. C., London. Caffeine and its Allies; Caffeine, Theine or Trimethylxanthine; Salts of Caffeine; Assay of Caffeine Sodium Salicylate; Theobromine; Diuretin; Derivatives of Caffeine; Theophylline; Tea; Constituents of Tea; Analysis of Tea; Moisture in Tea; Ash; Isolation and Estimation of Caffeine; Tannin; Extract; Stalks; Essential Oil; Adulterations of Tea; Caper Tea; Maté Paraguay Tea; Coffee; Composition of Coffee; Caffetannic Acid; Caffeoil; Coffee Berries; Analysis of Coffee and Coffee Mixtures; Coating and Glazing Substances; Ground Coffee; Commercial Chicory; Coffee Extracts; Kola; False Kola or Kola Bitter; Guarana; Bibliography.

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VOLUME VIII

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ANALYTICAL EXAMINATION OF THE VARIOUS
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EMPLOYED IN THE ARTS, MANU-
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WITH CONCISE METHODS FOR
THE DETECTION AND ESTIMATION OF THEIR IMPURITIES,
ADULTERATIONS, AND PRODUCTS OF DECOMPOSITION

VOLUME VIII

Glucosides, Non-Glucosidal Bitter Principles, Enzymes, Putrefaction
Bases, Animal Bases, Animal Acids, The Cyanogen
Compounds, The Proteins, The Digestion
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PREFACE

Allen's Organic Analysis, originally the work of one man on a relatively small scale, has now developed into a series of connected monographs by specialists in their respective fields, and there must inevitably be a certain amount of overlapping. Thus in the present volume the subject of enzymes falls into its appropriate place after the glucosides, but is also dealt with in connection with the hydrolytic dissociation of proteins.

The ideal arrangement would have been to have had all the sections on proteins in this volume, but want of space has prevented this, and sections dealing with certain classes of proteins and substances containing proteins have had, perforce, to be held over for Volume IX.

For the revision of those portions of the work for which in the last edition he was responsible, Dr. Schryver called in the assistance of his colleague, Dr. H. Buston, who ultimately, after Schryver's death, completed the work. These sections on the analysis of proteins therefore contain the last contributions of a great biochemist to the complex branch of chemistry which owes so much to his researches.

C. AINSWORTH MITCHELL.

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GLUCOSIDES

BY JULIUS GRANT, PH.D., M. Sc., A. I. C.

The term *glucoside* is applied to numerous substances possessing the common property of yielding dextro-glucose (dextrose) or an analogous compound of the sugar group as one of the products of hydrolysis on treatment with a dilute acid. Thus, salicin when boiled with dilute sulphuric acid is hydrolysed to dextrose and saligenin:



The majority of the glucosides yield dextrose, but many yield rhamnoses, fructose or galactose, and a few give remarkable sugars, such as apiose and digitoxose, of abnormal composition. Mannose is yielded only by strophanthin. In some cases the nature of the sugar has not been determined with certainty. Only 1 molecule of sugar usually results, though some glucosides contain 2 or more monosaccharides united as disaccharides or even as trisaccharides, and in a few cases they yield two different carbohydrates. These may sometimes be hydrolysed successively by the appropriate enzymes, with the production of new glucosides as the sugar components are gradually removed.

The natural glucosides are all products of the vegetable kingdom, and occur principally in fruits, barks, roots and plant pigments, but a few substances of animal origin are closely allied to them; these are the paired glucuronic acid derivatives which give rise to glucuronic acid, $\text{CHO}(\text{CH.OH})_4\text{CO}_2\text{H}$, instead of dextrose, on hydrolysis. The best known of these is euxanthic acid obtained from Purrée or Indian Yellow. A large number of substances, when introduced into the animal body, are excreted as paired glucuronic acid derivatives.

The function of glucosides in plant life is not yet fully understood. They may act as protectives or antiseptics, though cases are recorded

where they have proved toxic to their host (Sigmund, *Biochem. Z.*, 1914, **62**, 339). According to Armstrong they are more probably reserve materials, the gradual hydrolysis of which by a specific enzyme acts as a controlling medium for the plant metabolism and enables the products of hydrolysis to pass into circulation as required. Here they probably act as hormones or activators, by penetrating the plant cells and stimulating protoplasmic activity (H. E. and E. F. Armstrong, *Proc. Roy. Soc.*, 1910, **82**, [B], 588 *et seq.*; 1912, **84**, [B], 471). The glucoside content of the plant varies according to the season, and, in general, the glucosides in the seed are more complex in structure than those in the leaf. Many plant perfumes and pigments have been assigned a glucosidal structure (see p. 73).

Structure.—Pictet and Goudet found that, on distillation under reduced pressure, glucosides yielded *l*-glucosan, from which they inferred the presence of the 2:5-butylene ring structure. The general glucose ether structure $RO\cdot CH(CHOH)_2CH\cdot CHOH\cdot CH_2OH$,



in which *R* is an organic radical replacing a H atom of a —CHOH group of the glucose molecule, has therefore been assigned to them. According to whether the —OR group is on the same or opposite side of the molecule as the oxide grouping, so an α - or β -glucoside is obtained. These differ in physical and other properties. Thus, in general, the β -type only occurs in nature, whilst maltase and emulsin hydrolyse the α - and β -types only, respectively. The conversion of β - into α -glucosides has been effected by Helferich and Schneidmiller (*Ber.*, 1927, **60**, [B], 2002) and by Pascu, (*id.*, 1928, **61**, [B], 137). For the determination of the configuration of a glucoside, it thus is essential to know (1) whether it is of the α - or β -type, (2) the identity of the two components, and (3) the nature of the link connecting them. The structure cannot yet be related with physiological activity, though Jacobs and Hoffmann (*J. Biol. Chem.*, 1927, **74**, 787) have shown that the activity is reduced by saturation of the lactone linkage in certain cases.

Hydrolysis is usually effected by boiling with water under pressure, but it proceeds much more rapidly in presence of an acid. Many glucosides are very resistant to hydrolysis and require prolonged boiling with a fairly strong acid. Most glucosides are wholly unchanged when treated with alkali, but a few are decomposed.

Synthetic Glucosides.—A number of the natural glucosides have been synthesised, and in addition, a number obtained in the laboratory do not occur in plants. The principal methods employed are:—

(1) The condensation of dextrose and alcohol in the presence of hydrochloric acid (for glucosides of alcohols).

(2) Tetra-acetyl chlorodextrose, $C_6H_7OCl(OAc)_4$, is condensed with the sodium derivative of the alcohol or phenol—for helicin, methyl arbutin, etc. (Colley).

(3) Acetylbromodextrose is condensed with phenols (for α - and β -methyl glucosides), or with the silver salts of purines in the presence of quinoline or silver oxide and ether—for purine and mustard oil glucosides, prulaurasin, linamarin etc. (Fischer). Combination of the glucosides of purine bases with phosphoric acid has resulted in the production of compounds resembling nucleotides, (Fischer and Helferich, *Ber.*, 1914, 47, 210).

(4) Bargellini (*Gazz. Chim. Ital.*, 1914, 44, ii, 520) synthesised a number of chalkone glucosides by condensation of helicin with hydroxy derivatives of acetophenone in alkaline alcoholic solution.

(5) Enzyme synthesis. This important biological method depends on the principle enunciated by Bourquelot and Bridel (*Compt. rend.*, 1912, 155, 1552, *et. seq.*; 1915, 161, 184) that an enzyme which hydrolyses a glucoside in the presence of an excess of water will synthesise it in the presence of its hydroxy component. In particular, if the equilibrium attained by the enzymic hydrolysis of a glucoside to dextrose is upset by the addition of yeast (which destroys the dextrose), or of dextrose, then hydrolysis will be facilitated and retarded, respectively. A number of glucosides have been prepared and analysed in this way (see p. 4). It has been suggested by Rosenthaler that the production of an equilibrium is due to different enzymes acting in opposite directions.

Enzymes and Glucosides.—The glucosides are nearly always hydrolysed with relative ease at a low temperature, preferably at about 35°, by appropriate enzymes, which occur in the plant together with the glucoside. The best-known enzymes are emulsin of almonds and myrosin of mustard. Some of these enzymes are specific, their influence being exerted only on a few glucosides of closely related composition, and often they will hydrolyse a natural but not the same synthetic glucoside, and *vice versa*. Emulsin, however, decomposes a large number of glucosides of very different

composition, acting as it were as the master key of a variety of locks. It has been shown that almond emulsin is a mixture of several allied enzymes; this perhaps explains its catholic action. The effect of myrosin is connected with the presence of sulphur. The enzyme suitable for hydrolysis of a plant glucoside is present in the plant tissue, though in different cells, and reaction takes place only when these are ruptured, or their contents allowed to mix by the use of solvents. Armstrong showed that enzymes have great hydrolytic activity, even if they are insoluble in the medium used.

The measurement of enzymes in general is dealt with in the section on enzymes and a list of the principal glucosidoclastic enzymes follows:—

Enzyme	Principal substrates
Emulsin.....	Synthetic β -glucosides. Aesculin, amygdalin, arbutin, aucubin, coniferin, daphnin dhurrin, gentiopicrin, helicin, incarnatrin, indican, meliatin, prulaurasin, prunasin, salicin, sambunigrin, syringin, verbenalin, etc.
Myrosin.....	Sinigrin and <i>Sinapis</i> glucosides.
Amygdalase.....	Amygdalin.
Linase.....	Linamarin.
Prunase.....	Prunasin and similar glucosides.
Gaultherase.....	Gaultherin.
Rhamnase.....	Xanthorhamin.

Enzyme action has been used as a guide to the constitution of glucosides and for their synthesis (p. 3), and Kuhn and Sobotka (*Z. physikal. Chem.*, 1924, **109**, 65) have shown that *pH* value plays an important part in the control of these operations. Fischer showed that, in general, glucosides of phenols and phenol carboxylic acids are degraded by enzymes more easily than those derived from aliphatic alcohols and acids.

General Methods of Detection.—The arseno-tungstate reagent of Guglielmelli has been found suitable by Palet (*Anal. Soc. Quim. Argentina*, 1916, **4**, 256) as a test for hydroxyl groupings in glucosides. The detection and determination of hydrogen cyanide in cyanogenetic glucosides is dealt with on p. 14 *et seq.*

Enzyme Analysis, the principle of which has been stated above (p. 3) is of great importance. Thus a qualitative polarimetric test for plant glucosides is obtained if the change in optical activity

or reducing power, or both, towards Fehling's solution during enzymic hydrolysis is determined. When this change has ceased, the mixture is heated to 100° , cooled, and fresh enzyme added. If the rotation changes towards its original value, the presence of a glucoside is denoted. Bridel and Arnold (*Compt. rend.*, 1921, **172**, 1434) extract the plant with boiling alcohol, make the extract up to a definite volume with water, filter off the precipitate produced with lead acetate, remove the excess of lead with hydrogen sulphide, and evaporate the liquid under reduced pressure (below 50°). The residue is extracted in succession with ethyl acetate (to remove resins) and boiling 95% alcohol, and re-extracted with the latter after evaporation under reduced pressure with sodium carbonate. A solution of the residue in 100 c.c. of 50% methyl alcohol is digested with 0.5 grm. of emulsin at 20° , and the reducing sugars determined initially and every 10 days till there is no further decrease. The dextrose present originally may then be calculated. An allowance for conversion into β -methyl glucosides should be made. The glucoside itself may then be isolated. Ionescu defecates the original liquid with a 20% solution of trichloroacetic acid, and determines the dextrose in the filtrate by the potassium ferricyanide method. In the case of cyanogenetic glucosides the hydrogen cyanide (and benzaldehyde if it is present) must be removed by boiling. Bridel's method may be used in the presence of other sugars or, in the presence of lactose, Hildt's method (*Ann. Chim. Anal. Appl.*, 1920, **2**, 78) may be employed.

Ter Meulen observed the effect on enzyme hydrolysis of a glucoside of the addition of various sugars. The sugar which accelerates the reaction, instead of retarding it, is that present in the glucoside.

The great majority of the glucosides have a bitter taste. As a rule, they are neutral or faintly acid, only one or two basic plant principles (e.g., solanine, vernin) being glucosides. Their general properties are largely influenced by the non-sugar constituents, which are of a most diverse nature. Thus, the solubility varies greatly. Most of them are soluble in water and alcohol, insoluble in ether. Many are soluble in ethyl acetate and chloroform. Immiscible solvents also extract them from their acidified acid solutions, a behaviour which affords a general method of separating the glucosides from the stronger alkaloids, though not from weak bases, (see Plant Pigments, p. 76).

Name	Chief source	Formula	m. p., °C.	[α] _D	Sugar	Other constituents
Absothin.....	<i>Artemisia absinthium</i>	$C_{15}H_{20}O_4$	68	Dextrose.....	Volatile oil + aromatic resin.
Asculin.....	<i>Asculus hippocastanum</i>	$C_{15}H_{20}O_4$	205	Dextrose.....	Asculin $C_{15}H_{20}O_4$.
Agrostemma saponin.....	<i>Agrostemma githago</i> (corn cockle).	$(C_{15}H_{20}O_4)_2$	4 Sugars.....	$C_{15}H_{20}O_4$.
Amygdalin.....	Bitter almonds.	$C_{20}H_{27}O_{11}N$	200	-35	2 Dextrose.....	Benzaldehyde, hydrogen cyanide.
Amygdonitrile.....	<i>Cerasus padus</i> , <i>Prunus serotina</i> .	$C_{15}H_{19}O_8N$	147	-26.9	Dextrose.....	d-Mandelonitrile.
Androsin.....	<i>Apocyanum androsacmifolium</i>	$C_{15}H_{20}O_4$	218	Dextrose.....	Acetovanillone.
α-Antiarin.....	<i>Antiaris latifolia</i>	$C_{15}H_{20}O_4$	220	Antiarose.....	Antiarigenin.
Apin.....	<i>Apium petroselinum</i>	$C_{15}H_{20}O_4$	228	Apiose and dextrose.....	Apigenin.
Arbutin.....	<i>Arbutus uva ursi</i>	$C_{15}H_{20}O_7$	200	-60.5	Dextrose.....	Hydroquinone.
Aucubin.....	<i>Aucuba japonica</i>	$C_{15}H_{20}O_4$	180	-174	Dextrose.....	Aucubigenin.
Baptisin.....	<i>Baptisia tisclovia</i>	$C_{15}H_{20}O_4$	240	-61	2 Rhamnose.....	Baptigenin.
Barbaloin.....	Aloes	$C_{15}H_{20}O_4$	d-Arabinose.....	Alce emodin.
Calmatambin.....	<i>Canthium glabrifolium</i>	$C_{15}H_{20}O_{11}$	144	-130	Dextrose.....	Calmatambetin.
Campteritin.....	See Robinin.
Capsularin.....	<i>Corchorus capsularis</i>	$C_{15}H_{20}O_4$	175	-23.6	Dextrose.....	Capsularigenin $C_{15}H_{20}O_4$.
Castelin.....	$C_{15}H_{20}O_4$	205	62.9	Dextrose.....	Castelagenin.
Caulophyllosaponin.....	<i>Caulophyllum thalictroides</i>	$C_{26}H_{40}O_{17}$
Caulosaponin.....	<i>Ibid</i>	$C_{26}H_{40}O_{17}$	265	Dextrose.....	Centaureidin.
Cedrin.....	<i>Simaba cedron</i>	$C_{15}H_{20}O_4$	203	-85	Arabinose + galactose.....	Chydenanthigenin $C_{15}H_{20}O_4$.
Centaurein.....	<i>Centaurea jacea</i>	$C_{15}H_{20}O_4$	2 Dextrose.....	Mannitol.
Chydenanthin.....	<i>Chydenanthus excelsus</i>	$C_{15}H_{20}O_4$	198	-67.2	Dextrose and rhodose.....	Convulvulinic acid.
Clavicepsin.....	<i>Secale cornutum</i>	$C_{15}H_{20}O_4$	185	2 Dextrose.....	Cyanidin.
Coniferin.....	Conifers	$C_{15}H_{20}O_4$	150	Dextrose.....	Cyclamiretin $C_{15}H_{20}O_4$.
Convulvulin.....	<i>Ipomoea purga</i>	$C_{15}H_{20}O_{16}$	203	-22	Dextrose and cymarose.....	Strophanthidin.
Cyanin.....	Comflower and rose	$C_{15}H_{20}O_{12}$	252	23.5	Dextrose.....	Daphnetin.
Cyclamin.....	Cyclamen	$C_{15}H_{20}O_4$	139	Rhamnose.....	Datscetin.
Cymarin.....	<i>Apocyanum cannabinum</i>	$C_{15}H_{20}O_4$	200	2 Dextrose.....	Delphinidin + 2p-hydroxy-benzoic acid.
Daphnin.....	<i>Daphne genkwa</i>	$C_{15}H_{20}O_{11}$	190	Dextrose.....	p-Hydroxymandelonitrile.
Datscetin.....	<i>Datisca cannabina</i>	$C_{15}H_{20}O_{11}$	200
Delphinin.....	Larkspur	$C_{15}H_{20}O_{11}$
Dhurrin.....	<i>Sorghum vulgare</i>	$C_{15}H_{20}O_7N$	Dextrose.....	Benzoic acid.
Dibenzoylglucosylxose.....	<i>Dantesia latifolia</i>	$C_{26}H_{40}O_{17}$	148	Glucosylxose.....	Digitagenin.
Digitalin.....	<i>Digitaria latifolia</i>	$C_{26}H_{40}O_{17}$	227	Dextrose and digitalose.....	Digitogenin $C_{26}H_{40}O_{17}$.
Digitonin.....	<i>Ibid</i>	$C_{26}H_{40}O_{17}$	225	-50	4 Hexose + a pentose.....	Digitosapogenin.
Digitosaponin.....	<i>Digitaria purpurea</i>	$C_{26}H_{40}O_{17}$	Pentose.....	Digitoxigenin $C_{26}H_{40}O_{17}$.
Digitoxin.....	<i>Ibid</i>	$C_{26}H_{40}O_{17}$	256	2 Digitoxose $C_{15}H_{20}O_4$
Eldrin.....	See Rutin

Euxanthic acid (magnesium salt). Fatsin.	Mango leaves. <i>Fatsia japonica</i> .	$C_{10}H_{16}O_{10}$ $C_{11}H_{15}O_{10}$	Glucuronic acid. Dextrose.	Euxanthone. α - and β -sapogenins + organic acids.
Frangulin.	<i>Rhamnus frangula</i> .	$C_{10}H_{16}O_{10}$	228	Rhamnose.	Emodin.
Praxin.	<i>Fraxinus excelsor</i> .	$C_{10}H_{15}O_{10}$	320	Dextrose.	Fraxetin.
Rustin.	<i>Rhus colinus</i> .	$C_{10}H_{15}O_{10}$	218	Rhamnose.	2 Fisetin.
Galteolin.	<i>Galga officinalis</i> .	$C_{10}H_{15}O_{11}$	Dextrose.	Luteolin.
Gaultherin.	<i>Gaultheria procumbens</i> .	$C_{11}H_{15}O_{11}$	92	Dextrose + xylose.	Methyl salicylate.
Gentiaculin.	<i>Gentiana acaulis</i> .	$C_{11}H_{15}O_{11}$	Dextrose.	$C_{15}H_{19}O_4$.
Gentian.	Gentians.	$C_{15}H_{21}O_{14}$	274	Dextrose and xylose.	Gentienin.
Gentopictin.	<i>Ibid</i> .	$C_{15}H_{21}O_{14}$	191	Dextrose.	Gentienin.
Gitalin.	<i>Digitalis purpurea</i> .	$C_{15}H_{21}O_{14}$	155	Digitoxose.	Anhydrodigitaligenin, $C_{21}H_{25}O_8$.
Gitalin.	<i>Ibid</i> .	$C_{15}H_{21}O_{14}$	265	2 Galactose.	Digitogenin.
Gitalin.	<i>Ibid</i> .	$C_{15}H_{21}O_{14}$	272	3 Galactose + pentose.	Gitogenin $C_{21}H_{25}O_8$.
Gitalin.	Wallflower.	$C_{15}H_{21}O_{14}$	160	Dextrose.	Cherolin.
Glucocetrolin.	<i>Tropaeolum majus</i> .	$C_{15}H_{21}O_{14}$	175	Dextrose.	Benzyl mustard oil and $KHSO_4$.
Glucotropaeolin.	<i>Smilax glycophylla</i> .	$C_{15}H_{21}O_{14}$	Rhamnose.	Phloretin.
Glycyrrhizin.	Liquorice.	$C_{15}H_{21}O_{14}$	2 Glucuronic acid lactone.	Glycyrrhetic acid.
Gossypitrin.	<i>Gossypium herbaceum</i> .	$C_{15}H_{21}O_{14}$	162	Dextrose.	Gossypetin.
Gynocardin.	<i>Gynocardia odorata</i> .	$C_{15}H_{21}O_{14}$	72.5	Dextrose.	Hydrogen cyanide and $C_6H_5O_4$.
Gysophyllin.	<i>Gysophylla arrosif.</i>	$C_{15}H_{21}O_{14}$	Dextrose and galactose.	$C_{10}H_{15}O_4$.
α -Hederin.	<i>Hedera helix</i> .	$C_{27}H_{45}O_{11}$	256	Rhamnose + l-arabinose.	α -hederagenin $C_{41}H_{65}O_{14}$.
Hellcin.	(Yot natural).	$C_{27}H_{45}O_{11}$	174	Dextrose.	Salicyl aldehyde.
Hesperidin.	Citrus fruits.	$C_{28}H_{47}O_{11}$	151	2 Dextrose and rhamnose.	2 Hesperetin.
Hiptagenin.	<i>Hiptage madagabola</i> .	$C_{28}H_{47}O_{11}$	110	Dextrose.	Hiptagenin + 2 $C_6H_5O_4N$.
Hysyopin.	Ryssop.	$C_{28}H_{47}O_{11}$	275	Rhamnose.
Idain.	Cranberry.	$C_{28}H_{47}O_{11}$	Galactose.	Cyanidin.
Incarnatin.	<i>Trifolium incarnatum</i> .	$C_{28}H_{47}O_{11}$	242	Dextrose.	Quercetin.
Indican.	<i>Isatis tinctoria</i> .	$C_{28}H_{47}O_{11}$	100	Dextrose.	Indoxyl.
Iridin.	<i>Iris florentina</i> .	$C_{28}H_{47}O_{11}$	208	Dextrose.	Iugenin.
Isoquercitrin.	<i>Gossypium herbaceum</i> .	$C_{28}H_{47}O_{11}$	217	Dextrose.	Quercetin.
Jalopin.	<i>Conopodium scammonia</i> , <i>Jalapa</i> <i>oriza densis</i> .	$C_{41}H_{65}O_{14}$	131	3 Dextrose.	Jalopinolic acid.
Jegosaponin.	<i>Silyraz japonica</i> .	$C_{41}H_{65}O_{14}$	Dextrose.	Glucuronic and tiglic acids + 2 sapogenins, $C_{21}H_{25}O_8$, and $C_{21}H_{25}O_7$.

Name	Chief source	Formula	m. p., °C.	$[\alpha]_D^{20}$	Sugar	Other constituents
Levant sapotoxin.	See Gysophyllin					
Linamarin.	<i>Linum</i> species, <i>Phaseolus lina-</i> <i>ris</i> .	$C_{10}H_{17}O_6N$	142	-29	Dextrose.	Acetone cyanohydrin.
Loroglossin.	<i>Loroglossum hircinum</i> .	$C_8H_{14}O_6$	144	-45.65	2 Dextrose.	Loroglossigenin.
Lotusin.	<i>Lotus arabicus</i> .	$C_8H_{14}O_6N$			2 Dextrose.	Lotoflavin and hydrogen cya- nide.
Malvin.	Mallow.	$C_8H_{14}O_7$	165		2 Dextrose.	Malvin.
Melilotoside.	<i>Melilotus alissima</i> .	$C_{11}H_{19}O_7$	240	-64.1	Dextrose.	Coumaric acid.
Methyl arbutin.	<i>Arbutus uva ursi</i> .	$C_8H_{14}O_7$	175		Dextrose.	Hydroquinone methyl ether.
Monotropitin.	See Gaultherin					
Morindin.	<i>Morinda citrifolia</i> .	$C_8H_{14}O_{11}$	245		Dextrose.	Morindone (trihydroxy me- thyl anthraquinone).
Mowrin.	<i>Bassia longifolia</i> .				Lævulose + arabinose.	Mowric acid.
Myrtillin.	Whortleberry.	$C_8H_{14}O_6$			Dextrose.	Myrtillin.
Naringin.	<i>Citrus decumana</i> .	$C_{11}H_{19}O_7$	170		Dextrose and rhamnose.	Naringenin.
Nodakenin.	<i>Nodake</i> (<i>p. decursum</i>)	$C_8H_{14}O_6$	215	56.6	Dextrose.	Nodakenitin $C_{14}H_{14}O_4$.
Onidrin.	Blue grapes.	$C_8H_{14}O_6$			Dextrose.	Onidenin.
Oleandrin.	Oleander.	$C_{11}H_{19}O_7$	249		Digitalose.	Digitaligenin.
Orobanchin.	<i>Orobanchae rapum</i> .	$C_8H_{14}O_{12}$	180	-74.2	Dextrose + rhamnose.	Caffeic and other acids.
Quabain.	<i>Strophanthus graius</i> .	$C_8H_{14}O_{12}$	185	-31.5	Rhamnose.	A resin, $C_{21}H_{34}O_4$.
Pæonin.	Pæony.	$C_8H_{14}O_{14}$			2 Dextrose.	Pæonidin (cyanidin methyl ether).
Parillin.	<i>Smilax</i> .	$C_{11}H_{19}O_{10}$			2 Sugars.	Parigenin $C_{28}H_{46}O_4$.
Pelargonin.	<i>Pelargonium zonale</i> .	$C_{11}H_{19}O_{11}$	180		2 Dextrose.	Pelargonidin.
Phaseolunatin.	See Linamarin.					
Phloridzin.	Bark of apple, pear, cherry, plum.	$C_{11}H_{19}O_{10}$	170		Dextrose.	Phloretin.
Phytosterolin.	Olive bark, colocynth etc.	$C_{21}H_{41}O_6$			Dextrose.	A sitosterol.
Polydatoside.	<i>Polygonum cuspidatum</i> .		154	-57.9	Dextrose.	Polydatogenol.
Polygonin.	<i>Polygonum cuspidatum</i> .	$C_{11}H_{19}O_{10}$	202		Dextrose.	Emodin.
Populin.	Poplar.	$C_{11}H_{19}O_{11}$	180		Dextrose.	Saligenin and benzoic acid.
Primaverin.	<i>Primula officinalis</i> .	$C_{21}H_{41}O_{11}$			Dextrose.	Methyl β -methoxyxyresorcyate.
Primulaverin.	<i>Primula</i> .	$C_{21}H_{41}O_{11}$			Dextrose.	Methyl β -methoxyxyresorcyate.
Prulaurasin.	<i>Prunus laurocerasus</i> .	$C_{11}H_{19}O_{11}$	121	-52.7	Dextrose.	<i>dl</i> -Mandelonitrile.
Prunasin.	<i>Ibid.</i>	$C_{11}H_{19}O_{11}$	148		Dextrose.	<i>d</i> -Mandelonitrile.
Prunitrin.	<i>Prunus emarginata</i> .	$C_{11}H_{19}O_{11}$			Dextrose.	Prunetin.
Quercimeritrin.	<i>Gossypium herbaceum</i> .	$C_{11}H_{19}O_{11}$	245		Dextrose.	Quercetin.
Quercitrin.	<i>Quercus tinctoria</i> .	$C_{11}H_{19}O_{11}$	183	-141	Rhamnose.	Quercetin.
iso-Quercitrin.	<i>Gossypium herbaceum</i> .	$C_{11}H_{19}O_{12}$	217		Dextrose.	Quercetin.
Quillaic Acid.	<i>Quillaja Saponaria</i> .	$C_{11}H_{19}O_{10}$				
Quillaia Sapotoxin	<i>Ibid.</i>	$C_{11}H_{19}O_{10}$				
Quinovin.	<i>Ladenbergia oblongifolia</i> .	$C_{20}H_{40}O_5$		+59	Quinovose.	Quinovaic acid.

	<i>Rheum emodi</i>	$C_{11}H_{10}O_9$	230		Dextrose.	Rhapontigenin.
Rhapontin.	See Aucubin.		201		2 Rhamnose + dextrose.	Campferol.
Rhinanthin.	<i>Robinia pseudacacia</i>					
Robinin.	See Robinin.					
Robinoside.	See Robinin.					
Ruberythric acid.	<i>Rubia tinctorum</i>		258		2 Dextrose.	Alizarin.
Rubiadin.	Madder.	$C_{21}H_{20}O_9$			Dextrose.	Methyl xanthopurpurin.
Rutin.	Rue, etc.	$C_{27}H_{30}O_{14}$	190		Dextrose and rhamnose.	Quercetin.
Rutoside.	See Rutin.					
Sakuranin.	<i>Prunus pseudo-cerasus</i>		210		Dextrose.	Sakuranetin $C_{14}H_{14}O_8$.
Salicin.	Willow, poplar.	$C_{13}H_{14}O_7$	201	-65	Dextrose.	Saligenin.
Salungrin.	<i>Salix nigra</i>	$C_{13}H_{14}O_7$	195	-87.3	Dextrose.	m-Hydroxybenzaldehyde.
Sambunigrin.	<i>Sambucus nigra</i>	$C_{17}H_{14}O_9N$	151	-76.3	Dextrose.	l-Mandelonitrile.
Saponarin.	<i>Saponaria officinalis</i>	$C_{13}H_{14}O_7$			Dextrose.	Saponaretin.
Saporubin.	<i>Saponaria officinalis</i>				Dextrose and other sugars.	$C_{12}H_{14}O_8$
Sarsasapinin.	<i>Smilax ornata</i>	$(C_{13}H_{14}O_9)_4$	248	-54	3 Dextrose.	Sarsasapogenin $C_{34}H_{44}O_4$.
Scabiosin.	<i>Scabiosa succisa</i>	$C_{14}H_{16}O_{20}, 7H_2O$		-48.5	Dextrose.	Yellow solid.
Scammonin.	See Jalapin.					
Scillitin.	<i>Scilla maritima</i>	$C_{17}H_{24}O_4$	153		Dextrose.	Resins.
Scopolin.	<i>Scopolia japonica</i>	$C_{22}H_{28}O_{14}$	218		2 Dextrose.	Nonomethyl ether of aesculetin (scopoletin).
Serotin.	<i>Prunus serotina</i>		245		Dextrose.	Quercetin.
Sinalbin.	<i>Sinapis alba</i>	$C_{23}H_{30}O_{12}$	138		Dextrose.	Acrylylthiocyanate and sinapic acid sulphate.
		$C_{20}H_{12}O_{14}N_2S_2$				Allylthiocyanate + $KHSO_4$.
Sinigrin.	<i>Brassica nigra</i>		79	-16.1	Dextrose.	Skimmetin.
Skimmin.	<i>Stimmiia japonica</i>	$C_{10}H_{14}O_9NS_2K$	210		Dextrose.	Sophoretin.
Sophorin.	<i>Prunus serotina</i>	$C_{13}H_{16}O_{14}$			Dextrose + Rhamnose	Strophanthidin.
Strophanthin.	<i>Strophanthus Kombi</i>	$C_{20}H_{24}O_{16}$	171	29.2	Strophanthibiose methyl ether	Syringenin.
Syringin.	<i>Syringa vulgaris</i>	$C_{16}H_{24}O_9$	101	-17	Dextrose.	Quercetin + another glucoside.
Thulin.	<i>Thymus occidentalis</i>	$C_{21}H_{22}O_{11}$	184		Dextrose.	Ulexogenol.
Ulexoside.	<i>Ulex europaeus</i>		247	-51.9	Dextrose.	Uzardin $C_{14}H_{18}O_3$ + propyl alcohol.
Uzarin.	Uzara root.	$C_{17}H_{16}O_{20}$	210		3 Dextrose.	
						Guanine.
Vernin.	<i>Lupinus luteus</i>	$C_{10}H_{14}O_{14}N_2$	160	-40	d-Ribose	d-Mandelonitrile.
Vicianin.	<i>Vicia angustifolium</i>	$C_{15}H_{24}O_{10}N$	169	-30.2	Dextrose and arabinose	Methyl salicylate.
Violatosiside.	<i>Viola cornuta</i>				Dextrose + arabinose.	Rhamnetin.
Xanthorhamnin.	<i>Rhamnus species</i>	$C_{31}H_{42}O_{20}$			Galactose + 2 rhamnose.	

They are almost all lævorotatory, though derived from a dextrorotatory sugar. Some of them (digitalin, sapotoxin, strophanthin) are intensely poisonous. They crystallise with one or more molecules of water of crystallisation, which are usually removed progressively by heat. They char when heated to a high temperature, owing to the presence of the sugar component. This portion of the molecule does not reduce Fehling's solution.

A few glucosides are precipitated by alkaloid reagents, *e.g.*, tannin, picric acid, and some give characteristic colour reactions with acids, etc. Glucosides are best isolated by extraction with water, dilute alcohol or ethyl acetate after killing the plant enzyme to stop hydrolysis. The solution is clarified with lead acetate, the lead removed usually by hydrogen sulphide, and the filtered solution evaporated to the crystallising point. Sometimes it is quicker to steam-distil an aqueous extract of the bark, and to precipitate the distillate with lead chloride and lead oxalate. In most cases, owing to the small quantity present, the extraction is a matter of difficulty.

The classification of glucosides is not simple: they are most appropriately grouped according to the nature of the non-sugar component. On pages 6 to 9 is a tabular list of the better known glucosides arranged in alphabetical order; in many cases the information available is very scanty and probably needs revision.

Glucosides of Conifers

Coniferin, $C_{16}H_{22}O_8$, occurs in the cambium sap of coniferous trees, and is found also in beetroot and asparagus. It is readily prepared by evaporating the previously boiled and filtered juice to the crystallising point. It forms white satiny needles, often arranged in stellate groups, which contain $2H_2O$ and effloresce in dry air, become anhydrous at 100° , and melt at 185° . Coniferin is soluble in about 200 parts of cold water, but more readily in hot water and in alcohol. It is insoluble in ether. The aqueous solution of coniferin has a bitter taste, and is lævorotatory ($[\alpha]_D^{20} = -67.2^\circ$). Coniferin dissolves in strong sulphuric acid, with red colour, a deep blue resin separating on dilution with water. Moistened with phenol, and then treated with concentrated sulphuric or hydrochloric acid, it rapidly acquires a deep blue colour, the change occurring in sunlight almost instantaneously. By this reaction coniferin can be readily detected in pine

wood, and, conversely, pine wood moistened with hydrochloric acid may be used to detect phenols.

Coniferin gives no reactions with metallic solutions, and does not reduce Fehling's solution. Chromic acid mixture oxidises it to vanillin. It is gradually hydrolysed by emulsin into dextrose and coniferyl alcohol. If dilute acid is used as the hydrolysing agent, the coniferyl alcohol becomes polymerised to a resinoid substance. A number of its derivatives have been described by Odén (*Chem. Soc. Abs.*, 1920, i, 247), and it has been synthesised by Pauley and Fenerstein (*Ber.*, 1927, 69, [B] 1031) from hydroxy-cinnamaldehyde.

Syringin, $C_{17}H_{24}O_9 + H_2O$, occurs in the syringa and in the bark of lilac and privet. It crystallises in long slender colourless needles, sparingly soluble in cold water, more readily in hot. It becomes anhydrous at 100° and melts at 191° ; it has $[\alpha]_D - 17^\circ$. With mineral acids it reacts similarly to coniferin. Emulsin hydrolyses it to dextrose and syringenin, *i.e.*, dimethoxyconiferyl alcohol. The formula of hydrated syringin, $C_{17}H_{26}O_{10}$, is the same as that of the saponins, but it has no relation to these.

Glucosides of Willow and Poplar

Salicin, $C_{13}H_{18}O_7$, occurs in the bark and leaves of many, but not all, species of *Salix* (willow) and *Populus* (poplar), and also in the female flowers and flower buds of meadow-sweet (*Spiraea ulmaria*). The amount varies according to the time of the year, and is usually highest in autumn. Clark and Gillie (*Amer. J. Pharm.*, 1921, 93, 618) have studied the amounts found in various sources.

Salicin is *o*-hydroxybenzyl glucoside, $C_6H_4(OC_6H_{11}O_6).CH_2(OH)$. It is hydrolysed slowly by dilute mineral acids, very readily by the emulsin of almonds and by an enzyme called *salicase* present in willow leaves and twigs, to dextrose and saligenin, $C_7H_8O_2$, which is *o*-hydroxybenzyl alcohol. Its benzoyl derivative is populin (*vide infra*).

It is prepared by exhausting willow bark with boiling water, concentrating, precipitating impurities with lead acetate equal to 10% of the bark taken, and freeing the filtrate from lead. It is then concentrated *in vacuo* and left to crystallise. The impure salicin is recrystallised from alcohol and decolorised with animal charcoal. It crystallises in needles, plates or rhombic prisms, m. p. 198 to 201° , and decomposes at 240° ; in aqueous solution it has $[\alpha]_D^{15} - 65^\circ$.

Salicin has a bitter taste, and, since it possesses febrifugal properties, is used for rheumatism. It dissolves sparingly in cold (1:28), very readily in hot water (1:0.7), and dissolves in alcohol (1:30 cold, 1:2 boiling). It is insoluble in organic solvents.

According to the British Pharmacopœia salicin (salicinum) is neutral, soluble in 28 parts of water and 80 parts of 90% alcohol, but not in chloroform or ether, and oxidation of 0.1 gm. with 0.2 gm. of potassium dichromate and 2 c.c. of sulphuric acid should produce an odour of meadowsweet (salicyl aldehyde). The U. S. Pharmacopœia, also stipulates a solubility of 1 gm. in 23.5 and 88.5 grms. of water and alcohol, respectively, at 25°. In addition, salicin should be free from heavy metals, (ash not more than 0.05%), and 10 c.c. should not be precipitated by tannin, neutral or basic lead acetate, or by alkaloid reagents. It should give a red colour with ferric chloride (due to salicylic acid) only after it has been just melted, and it should dissolve in concentrated sulphuric acid and produce an intense red coloration which disappears on the addition of water—the so-called "*rutilin reaction*."

Saligenin crystallises in small tables m. p. 82°. It is soluble in cold water (1:15), alcohol, ether and benzene and is characterised by an indigo-blue coloration with ferric chloride. Ether extracts it from aqueous solutions.

To estimate salicin it should be hydrolysed by emulsin at 37°, and the dextrose which is produced determined. As a rough check, the saligenin may be extracted with ether and weighed.

Populin, $C_{20}H_{22}O_8$, or benzoylsalicin, in which the benzoyl group is attached to the sugar nucleus and not to the alcohol group of saligenin, occurs in the bark and leaves of the aspen and other poplars and is obtained from salicin by benzylation. It forms delicate needles, m. p. 180°, and is very sparingly soluble in cold, more soluble in boiling water (1:42). Barium hydroxide hydrolyses it to benzoic acid and salicin; dilute acids form benzoic acid, dextrose and saliretin, the condensation product of saligenin. Emulsin is without action. It is coloured bright red by concentrated sulphuric acid.

Salinigrin, $C_{13}H_{16}O_7$, has been found only in *Salix discolor* out of 33 samples of willow and poplar examined by Jowett and Potter, (*J. Chem. Soc.*, 1900, 77, 707). It has m. p. 195°, $[\alpha]_D - 87.3^\circ$, and gives no coloration with sulphuric acid. It is an

isomer of helicin, since *m*-hydroxybenzaldehyde is formed on hydrolysis.

Helicin, $C_{18}H_{16}O_7$, the glucoside of *o*-hydroxybenzaldehyde, $C_6H_4(OC_6H_{11}O_5).CHO$, does not occur naturally, but is obtained by the oxidation of salicin with dilute nitric acid; it was prepared synthetically by Michael from acetochloro-glucose and salicylaldehyde. Nascent hydrogen reduces it to salicin. It crystallises in bunches of slender needles, m. p. 174° , and emulsin hydrolyses it, and also its hydrazone and oxime. Clark (*loc. cit.*, p. 99) has described a number of its derivatives.

Other Phenol Glucosides—Arbutin, $C_{12}H_{16}O_7$, occurs with *methyl arbutin*, $C_{13}H_{18}O_7$, in *Arbutus uva ursi*, and the latter is always present in commercial preparations of the former. It may be removed by precipitation of the arbutin by potassium hydroxide from a solution in alcohol, and decomposition of the washed and filtered precipitate by sodium carbonate (Hérissey); or by separation of the addition compound formed by arbutin and hexamethylene tetramine (Mannich). Both methods are imperfect. Arbutin behaves as a diuretic by virtue of its hydrolysis product, hydroquinone. Both glucosides are hydrolysed by emulsin, though the rate of reaction is slow in the case of arbutin, and the hydroquinone produced is dark in colour as a result of enzymic oxidation. The latter fact has been held responsible for the darkening in colour of the leaves of certain varieties of *Pyrus*. *Methyl arbutin* was synthesised by Michael from acetochloro-glucose and hydroquinone methyl ether.

Phloridzin, $C_{21}H_{26}O_{10}$, occurs in the barks of the apple, plum and cherry trees and other *Rosaceæ*. It produces glucosuria, and is hydrolysed by mineral acids (but not by emulsin) to phloretin and dextrose. Phloretin is a condensation product of phloroglucinol and *p*-hydroxy atropic acid, and also occurs in glycyphyllin, the glucoside of *Smilax glycyphylla*. Its derivatives are described by Odén (*loc. cit.*, p. 11).

Gaultherin, $C_{14}H_{18}O_8$, is widely distributed, but its principal source is *Gaultheria procumbens*. It is a methyl salicylate glucoside and is hydrolysed by acids or by a specific enzyme, gaultherase, but not by emulsin. The synthetic form prepared by Karrer from diazomethane and glucosidosalicylic acid is, however, hydrolysed by emulsin and not by gaultherase, and though this indicates that

a β -glucoside had been produced, it does not necessarily follow that the natural product is an α -glucoside, (*Helv. Chim. Acta.*, 1920, 3, 252).

Aucubin, $C_{13}H_{20}O_8$, from *Aucuba japonica* is hydrolysed to dextrose and aucubigenin, which is responsible for the discoloration of spotted laurel leaves.

Cyanogenetic Glucosides

Glucosides yielding hydrogen cyanide on hydrolysis, of which amygdalin is a well-known example, have been occasionally found in plants used as fodder, and their detection thus becomes a matter of some importance. They are known as cyanogenetic or cyanophoric glucosides, and are of theoretical importance, in that they were among the first to be synthesised.

It suffices, as a rule, to macerate the plant tissue with water at about 30° in a closed vessel and then to remove the hydrogen cyanide formed, by distillation, though owing to frothing the aqueous liquid is often difficult to distil. Hydrogen cyanide can be detected by any of the well-known tests, the formation of a rose-red coloration with sodium picrate paper¹ and of a blue colour with a benzidine and copper acetate paper in the presence of a drop of toluene or chloroform being among the most sensitive, and determined quantitatively by modifications of the ordinary methods. A useful microchemical method of detection which depends on the catalysis of ammonia and alloxan by hydrogen cyanide, due to Denigès (*Ann. Chim. Anal.*, 1921, 3, 179), may also be mentioned. Two c.c. of nitric acid (sp. gr. 1.38) and 2 grm. of uric acid are shaken till no more red fumes are evolved, 2 c.c. of water added, and the mixture boiled and made up to 100 c.c. One drop of this solution on a microscope slip is mixed with a small drop of ammonia, or pyridine, and quickly inverted over the mouth of a test-tube containing the sample (e.g., the plant macerated with water). If the reagents are fresh, 0.0001 mg. of cyanide is detectable from the characteristic crystals produced.

The procedure adopted by Henry and Auld (*J. Soc. Chem. Ind.*, 1908, 27, 428) depends on the partial isolation of the glucoside and its decomposition by boiling with mineral acids. The product is

¹ Slips of filter-paper are soaked in a solution of 5 grm. of picric acid, and 50 grm. of sodium carbonate per litre, drained, and dried.

ground as rapidly as possible, weighed, placed in a Soxhlet extraction apparatus and percolated with hot alcohol to dissolve out the glucoside. The solvent is distilled off, the residue mixed with 50 c.c. of water, and 10 c.c. of 10% hydrochloric or sulphuric acid added. The mixture is then distilled, preferably in a current of steam, until hydrogen cyanide can no longer be found in the distillate, in which it may be estimated volumetrically by Liebig's method. A slight excess of sodium hydrogen carbonate is preferably added, and the solution titrated with an excess of iodine solution. A little of the freshly ground product is macerated with water in presence of an antiseptic to ascertain whether hydrogen cyanide is formed, and thereby the presence of the enzyme is denoted.

Later work has shown that when more exact determinations of hydrogen cyanide are required, numerous precautions are necessary in the above method. In the first place, distinction must be drawn between (1) naturally-occurring free hydrogen cyanide, (2) that in non-glucosidal combination (e.g., as $C_6H_5COOH.HCN$), and (3) that produced from the glucoside. A 5% solution of mercuric chloride may be used to fix (as mercuric cyanide) free hydrogen cyanide or that held in non-glucosidal combination, and the gas may subsequently be liberated by the action of carbon dioxide on a solution of the precipitate in potassium iodide solution, and its amount deducted from the total figure obtained by Liebig's method. The gas also combines with dextrose to form ammonium glucoheptonate, $C_6H_{13}O_6$, $COOK.NH_3$. On the other hand, prolonged maceration may cause fixation of hydrogen cyanide, whilst incomplete hydrolysis will also produce low results, and a balance must be maintained between these sources of error. The acid is also evolved very slowly during distillation. Morris (*Amer. J. Pharm.*, 1920, 92, 908) states that the use of borax during the titration, in place of sodium hydrogen carbonate, enables a more accurate adjustment of the alkalinity to be obtained. In cases where the products of hydrolysis may interfere with the titration, Furlong (*Analyst*, 1914, 39, 430) recommends a colorimetric method (see p. (3) 16).

The A.O.A.C. (*Official and Tentative Methods of Analysis of the American Association of Official Agricultural Chemists*, 1925) recommends the following tentative methods for cyanogenetic beans.

(1) *Acid Titration*.—The powder (10–20 grm.), ground to pass a 20-mesh sieve, is macerated in a flask for 2 hours with 100 c.c. of

water, a further 100 c.c. added, and 150 c.c. of the total mixture steam-distilled into 20 c.c. of 0.02*N* silver nitrate solution and 1 c.c. of nitric acid. An adapter should be used, but the addition of paraffin wax to prevent bumping should be avoided, as it fixes a portion of the gas. After removal of the precipitate by filtration, the excess of silver nitrate is titrated with 0.02*N* potassium thiocyanate solution, with ferric alum as indicator. Then 1 c.c. of $\text{AgNO}_3 = 0.54$ mg. of HCN.

(2) *Alkaline Titration*.—The distillate [see (1)] is collected in 20 c.c. of a 2.5% solution of sodium hydroxide and titrated with 0.02*N* silver nitrate solution till the first permanent turbidity is produced.

(3) *Prussian Blue Method*.—The distillate is collected as in Method (2), diluted to 200 c.c., 20 c.c. pipetted out, and concentrated to 1 c.c. *in vacuo* below 70°, and 0.4 c.c. of a fresh 3% solution of ferrous sulphate and 0.5 gm. of potassium fluoride added. The container is at once exhausted, to prevent oxidation, the contents mixed for 10 minutes, and the blue colour, which is produced at 50° after the addition of 30% of nitric acid, matched against a standard prepared in a similar fashion from a solution containing 10 mg. of potassium cyanide in 250 c.c. of water.

A gravimetric modification is due to Lührig (*Chem. Ztg.*, 1920, **44**, 166), in which the precipitate produced from the distillate and the silver nitrate is treated with ammonia, and any insoluble silver sulphide (produced from sulphur compounds) filtered off. The silver cyanide in the filtrate is precipitated by nitric acid, ignited and weighed as silver.

Lavialle and Varenne (*J. Pharm. Chem.*, 1918, **17**, 97) produce ferric thiocyanate, which may be matched colorimetrically as follows:—The solution is evaporated on a water-bath, a yellow colour being maintained by requisite additions of calcium polysulphide (prepared by bubbling hydrogen sulphide through 20 gm. of pure lime in 100 c.c. of water for 15 minutes, followed by filtration, the addition of 5 gm. of powdered roll-sulphur, and a further 15 minutes on the water-bath). The residue is extracted with 5 c.c. of water and 5 drops of 20% sulphuric acid, and a slight excess of calcium carbonate added. The filtered liquid is evaporated, re-filtered if more carbonates or calcium sulphate, or both, separate, and the residue dissolved in 1.0, 0.5 or 0.25 c.c. of water, and 4, 2

or 1 drops of sulphuric acid added, respectively. The exact acidity is important. Ferric sulphate solution (5%) is then added till there is no further increase in the red colour, which may then be matched against a standard, or titrated with a 0.01*N* solution of silver sulphate till the colour disappears. The blank on the reagents must be determined, and an accuracy of 5% is obtainable for 0.01 mg. of hydrogen cyanide. Menaul and Dowell (*J. Agric. Res.*, 1920, **18**, 44) obtain a more permanent colour by removal of the sulphide by the addition of calcium chloride to an acid solution before the addition of the iron salt.

Roe's method (*J. Biol. Chem.*, 1924, **58**, 667), in which hydrolysis is effected by emulsin, and the errors due to distillation thereby avoided, is stated to be particularly suitable for cyanogenetic glucosides in a relatively pure state, since soluble cyanides are also included in the result. The sample of glucoside (0.1 grm.) is shaken at 45° for 15 minutes or in a stoppered flask with 100 c.c. of water, 0.05 grm. of emulsin and a few drops of amyl or capryl alcohol, and then connected with a flask, attached to a suction-pump, and containing 150 c.c. of a 5% solution of sodium hydroxide. The former flask is aerated at the rate of 3 litres per minute for 3 hours, and the hydrogen cyanide drawn over into the alkali, which is then titrated with 0.01*N* silver nitrate solution, in the presence of 10 drops of a 10% solution of potassium iodide, till a faint permanent turbidity appears. The factor 0.5404 gives the hydrogen cyanide in mg. Bishop (*Biochem. J.*, 1927, **21**, 1162) confirms the superiority of this procedure over distillation. Direct extraction of the hydrogen cyanide from the plant by solvents such as potassium nitrate and formaldehyde has also been attempted (Menaul, *J. Biol. Chem.*, 1921, **46**, 297), but yields unreliable results.

Since Henry and Auld found 0.032 and 0.045% of hydrogen cyanide in two samples of linseed cake, but none when the cake was ground with water, it is probable that the enzyme of linseed is destroyed during the hot expression of the oil, so that the manufactured cake is not toxic. Cases are recorded, however, where it has caused cattle poisoning, (Cranfield, *Analyst*, 1925, **50**, 18), though there is also contrary evidence that cyanogenetic glucosides may be excreted unchanged.

Reichard (*Z. Unters. Nahr. Genussm.*, 1924, **47**, 339) also points out that, since the fining of wine with ferrocyanides is now permitted

in Germany, the presence of hydrogen cyanide is not necessarily evidence of the use of stone fruit.

Numerous cases of the poisoning of cattle by Java beans (*Phaseolus lunatus*) are on record. Henry and Auld find that dark-coloured Java or Mauritius beans yield more hydrogen cyanide than the lighter coloured Burma beans, whilst the cultivated large white beans produced in Madagascar, France, and the United States usually furnish only traces of the acid, less than 0.02% being the amount tolerated in most cases (see Kohn-Abrest, *Ann. Falsif.*, 1917, 10, 17). The colour of the beans must not, however, be correlated with the amount of acid that they may produce.

A cyanogenetic glucoside also occurs in sorghum and in the stem and tubers of bitter and sweet cassava. These plants almost always contain a cyanogenetic glucoside and the corresponding enzyme, but free hydrogen cyanide is not present. The function of the hydrogen cyanide is obscure, but it probably plays a part in nitrogen assimilation by the developing plant. Certain common grasses (e.g., *Tridens flavens*) are also cyanogenetic, and Petrie (*Chem. News*, 1914, 110, 126) found 20 grasses among the 60 plant species of New South Wales stated to have these properties.

The isomeric amygdalin glucosides may be classified according to the optical properties of the phenylglycollic acid produced after hydrolysis by hot concentrated hydrochloric acid.

Monoglucosides $C_{14}H_{17}O_6N$	Diglucosides $C_{20}H_{27}O_{11}N$	Rotation
Prunasin	<i>l</i> -Amygdalin (glucoprunasin)	<i>l</i> -
Prulaurasin	<i>iso</i> -Amygdalin (gluco-prulaurasin)	<i>i</i> -
Sambunigrin	<i>d</i> -Amygdalin (glucosambunigrin)	<i>d</i> -

Amygdalase or an extract of bottom-fermentation yeast converts the di- into the corresponding mono-glucosides, whilst the action of alkali converts the optically active mono- or di-glucosides into their respective *dl*-equivalents. The hydrolysis of these glucosides by emulsin is due to the successive actions of two separate enzymes (amygdalase and prunase) contained in the latter, which can act only in the order mentioned. Many of these glucosides have been synthesised by Fischer and Bergmann (*Ber.*, 1917, 50, 1047) and by Campbell and Haworth (*J. Chem. Soc.*, 1924, 125, 1337).

The best known cyanogenetic glucosides are:—

Amygdalin, $C_{20}H_{27}O_{11}N$, m. p. 200° , is extracted by means of 70% alcohol from bitter almonds or from the kernels of peaches, cherries, plums, apples, etc. (*i.e.*, *Rosaceæ* fruits). It is hydrolysed by emulsin or by hot dilute hydrochloric acid to hydrogen cyanide, benzaldehyde, and 2 molecules of dextrose, and is thus a source of bitter almond flavour. It occurs in colourless crystals having a bitter taste, and though it has been the object of many investigations (see Armstrong, *Bibliography*), its structure is not yet completely known. The intermediate compounds produced during the enzymic hydrolysis of amygdalin have also been the subject of a number of investigations (see Rosenthaler, *Arch. Pharm.*, 1925, **263**, 563). Several amygdalin derivatives are described by Odén (*loc. cit.* p. 11).

The Oleum Amygdalæ Amaræ, or oil of bitter almonds, of the British and U. S. Pharmacopœias is described in the latter as the neutral volatile oil obtained by maceration and steam distillation of the ripe kernels (free from fixed oil) of *Amygdalus communis* L. It should not crystallise and should yield not less than 85% of benzaldehyde and 2–4% of hydrogen cyanide; sp. gr. 1.038–1.060 at 25° ; $[\alpha]_D$ zero to 10° ; refractive index (20°) 1.5428–1.5439. The fixed oil expressed from the seeds, however, is odourless and has a bland taste.

d-Amygdonitrile glucoside (Prunasin), $C_{14}H_{17}O_6N$, m. p. $147-150^{\circ}$, was first obtained from amygdalin by the action of dried extract of bottom-fermentation yeast. It has since been found in *Cerasus padus* and *Prunus scrotina*. It has $[\alpha]_D - 27^{\circ}$ and is hydrolysed to dextrose and d-mandelonitrile.

Sambunigrin, $C_{14}H_{17}O_6N$, m. p. 151° , is present in the leaves of the elder, *Sambucus niger*. It has $[\alpha]_D - 76^{\circ}$, is hydrolysed by emulsin to dextrose and l-mandelonitrile, and was synthesised by Fischer and Bergmann (*Ber.*, 1917, **50**, 104).

Prulaurasin (Laurocerolin), $C_{14}H_{17}O_6N$, m. p. 122° , occurs in the leaves of the common cherry laurel, *Prunus laurocerasus*; it has $[\alpha]_D - 52.75^{\circ}$ and yields dextrose and dl-mandelonitrile on hydrolysis. It is obtained from the preceding glucosides by the action of alkali.

Linamarin (Phaseolunatin) $C_{10}H_{17}O_6N$, m. p. 142° , $[\alpha]_D - 291$ has been obtained from the rubber tree, from flax, cassava and the beans of *Phaseolus lunatus*. It is hydrolysed to dextrose and ace-

tone cyanohydrin. It is the β -glucoside of acetone cyanohydrin and contains an enzyme linase, and one similar to amygdalase. It was synthesised by Fischer and Anger.

Lotusin, $C_{28}H_{31}O_{16}N$, is obtained from *Lotus arabicus*, an Egyptian fodder plant. It is hydrolysed to 2 molecules of dextrose, hydrogen cyanide and lotoflavin, (a colouring matter of the quercitin group, and an isomer of fisetin), by acids, or by the enzyme lotase.

Dhurrin, $C_{14}H_{17}O_7N$, is present in the great millet, *Sorghum vulgare*. On hydrolysis by emulsin, dextrose, hydrogen cyanide and *p*-hydroxy-mandelonitrile are obtained.

Gynocardin, $C_{13}H_{19}O_9N$, m. p. 162° has been found in *Gynocardia odorata* and *Pangium edule*. It forms dextrose, hydrogen cyanide and a complex substance of the formula $C_6H_8O_4$ when hydrolysed by gynocardase or acids.

Vicianin, $C_{19}H_{25}O_{10}N$, m. p. 160° , $[\alpha]_D - 20^\circ$, occurs in the seeds of *Vicia angustifolia*. It is decomposed by an enzyme (vicianase) present in certain vetches into hydrogen cyanide, benzaldehyde and a disaccharide, *vicianose* ($C_{11}H_{20}O_{10}$) which is further hydrolysed by almond emulsin to dextrose and *l*-arabinose (Bertrand, *Compt. rend.*, 1910, **151**, 325). Thus, it may be regarded as amygdalin in which dextrose is replaced by arabinose.

Glucosides of Mustard

Black and white mustard, the seeds of *Brassica* or *Sinapis nigra* and *alba* contain the glucosides *sinigrin* and *sinalbin*, together with an enzyme *myrosin*. They also contain sinapin thiocyanate, the proportion of this being much larger in the white seeds. The commercial ground mustard is a mixture of the two varieties. Both plants belong to the family *Cruciferae* and are erect annuals. Indian mustard is obtained from *B. juncea*, Russian mustard from *B. Besseriana*, and Dakota or Charlock mustard from *B. sinapistrum*.

Black or brown mustard seeds are spherical in shape, very small, about 50 or 60 weighing a grain, of a dark reddish-brown colour outside and yellow within. The surface is reticular and full of small depressions; when crushed and moistened the seeds have a pungent taste and odour.

White mustard seeds are yellow in colour, distinctly larger than the black, about 10 weighing a grain, and the surface is smoother. They

remain inodorous when crushed and moistened, but have a pungent taste.

Microscopic Structure.—The seed proper consists entirely of minute oil-bearing cells which look very like starch granules, but neither give a blue colour with iodine nor polarise light. The husk is very complicated, being built up of no less than six layers. The outer layer is transparent and consists of a single layer of large hexagonal cells, filled with mucilage, which swell up and are ruptured when immersed in water. Beneath this are two layers of large cells the walls of which are thickened at the angles, and a single layer of small angular cells, called palisade cells, the walls of which contain most of the yellow colour of the husk. The inner layers consist of a colourless parenchyma resting on the aleurone layer of large polygonal cells with fat globules and granular protein matter. In the black mustard, the palisade cells are of unequal length and are superposed on one or two layers of pigment cells.

A slide of ground mustard shows granular masses of loose fine grey texture, globular oil drops, with patches here and there of the yellow layer and of the hexagonal mucilage cells. (Diagrams of mustard are given by Clayton, "A Compendium of Food Microscopy," by Leach, "Food Inspection and Analysis," and Greenish "Food and Drugs.")

Black mustard seeds contain fixed oil, (31 to 37%), sinigrin and myrosin, and the seed coats contain a soluble mucilage. Starch is absent from the ripe seed, though present in unripe mustard. The seeds yield about 0.5% of volatile oil. They differ from rapsin seed in that the skin of the latter is smooth and not crossed by ribs forming hexagonal hollows. A section shows cells of the same height, taller than they are wide, whilst those of mustard seed show a single stratum of almost square cells graduated in size.

White mustard seeds contain fixed oil, (25 to 26%), sinalbin, myrosin, sinapin thiocyanate and mucilage; starch is not present.

Fixed Oil.—The oil of black mustard seed has a brownish-yellow colour and a mild taste; it closely resembles rape oil in chemical composition. Sp. gr. at 15°, 0.916 to 0.920; solid. pt. —17°; saponification value, 173 to 175; it contains the glycerides of erucic and stearic acids and a liquid fatty acid. By the lead salt and ether method it yields 2.3 to 4% of solid fatty acids. According to Lewkowitsch it is used for soap making and is not suitable for burning.

White mustard oil is almost identical with black mustard oil, but the iodine value is lower and it yields only traces of solid acids. It is used as a burning and lubricating oil.

Both oils have mild rubefacient properties. Raynes points out (*Analyst*, 1918, **43**, 216) that the iodine values given by Lewkowitsch for these oils are lower than those found by many other workers, and suggests the limits for the expressed black and white mustard oils of 119.6–121.0, 114.4, and 104.7–108.6, respectively.

Glucosides.—To isolate *sinalbin*, white mustard seed should be finely ground and carefully freed from fixed oil by pressure or by extraction with benzene or carbon disulphide. The dry powder is then extracted for half an hour with four times its weight of boiling alcohol and filtered hot; on cooling, sinalbin crystallises out.

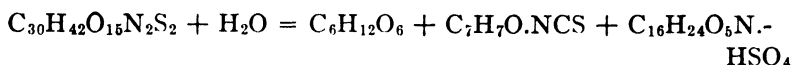
To prepare *sinigrin*, black mustard seed is similarly extracted with boiling alcohol, after careful removal of the fixed oil by suitable solvents. With both varieties of mustard it is advisable to add the powdered seed to the boiling alcohol so as to destroy the myrosin immediately. The extract is evaporated to dryness and the residue extracted with cold water. The aqueous solution is evaporated with the addition of a little barium carbonate, and the residue taken up with boiling spirit. Sinigrin crystallises after a time from this solution. Hérissé and Boivin (*Bull. Soc. Chim. Biol.*, 1927, **9**, 947, 950) obtained a yield of 12 grm. from 1 kgrm. of black mustard seed by extraction with boiling 25% acetone for 20 minutes, the cooled, filtered extract being concentrated, re-filtered and fermented with bakers' yeast for 2–3 days. The glucoside was extracted with alcohol from the fermented solution after neutralisation with calcium carbonate, filtration and evaporation. To obtain free myronic acid, sinigrin is mixed with tartaric acid and alcohol; the acid potassium tartrate is removed by filtration, and the filtrate evaporated to a syrup. Myronic acid has a strong acid reaction and readily decomposes.

Sinigrin or potassium myronate, $C_{10}H_{16}O_9NS_2K$, crystallises in compact, lustrous colourless needles (m. p. 79° , $[\alpha]_D - 16.13$), very soluble in water, sparingly soluble in cold alcohol. It is hydrolysed by myrosin to dextrose, mustard oil or allyl isothiocyanate (see p. 23) and potassium hydrogen sulphate.



On the addition of potassium methoxide, potassium sulphate separates at once.

Sinalbin, $C_{30}H_{42}O_{15}N_2S_2 \cdot 5H_2O$, crystallises in faintly yellow coloured needles, m. p. 138 to 140° , slightly soluble in cold, readily in hot water. It is hydrolysed by myrosin to dextrose, sinalbin mustard oil and sinapin hydrogen sulphate.



Sinalbin mustard oil is *p*-hydroxybenzylisothiocyanate; it is not volatile and is therefore destitute of pungent odour or effect on the eyes. Sinapin hydrogen sulphate is converted by barium hydroxide into choline and sinapic acid, $C_6H_2(OH)(OMe)_2.CH:CH.CO_2H$.

Sinapin, $C_{16}H_{23}O_5N$, exists as thiocyanate in white (and black) seeds, and as sulphate in the glucoside sinalbin. The free base is known only in solution: to obtain it the thiocyanate is treated with silver sulphate and the sulphate precipitated with baryta. It has an intense yellow colour in aqueous solution and is strongly alkaline. On evaporation it decomposes into choline and sinapic acid; with acids it forms crystalline salts. The thiocyanate crystallises in colourless needles (m. p. 176°) which are yellow if impure; it gives a red coloration with ferric chloride. It has been synthesised by Späth (*Monatsh.*, 1920, **41**, 271).

Myrosin is prepared by extracting ground white mustard seed with cold water and precipitating with strong alcohol. The aqueous extract may be concentrated *in vacuo* below 40° , if desired. The precipitated enzyme is soluble in water; the aqueous solution is coagulated by heat and has the general properties of enzyme solutions. It is very active toward sinigrin and sinalbin. In the case of moderately concentrated solutions of sinigrin, as action proceeds, the potassium hydrogen sulphate formed renders the enzyme less active and finally inactive.

The amount of myrosin contained in black mustard seed is said usually to be insufficient to act on all the glucoside present, whilst white mustard seed contains an excess of myrosin. It is therefore customary to mix the two varieties.¹ Myrosin has its maximum activity at a little below 50° . It is without action on amygdalin and

¹ Greenish (*Pharm. J.*, 1912, 203) has proved that black mustard seeds contain more than sufficient myrosin.

on phenolic- β -glucosides, and is detectable by Peche's method, in which a section of the plant is immersed in a 1% solution of osmic acid and then in a 10% solution of potassium myronate saturated with barium chloride. The formation of white globules of barium sulphate on the cells can be seen as hydrolysis proceeds. If calcium or strontium chlorides are used the crystals are needle-shaped and granular, respectively. Myrosin is widely distributed in cruciferous plants, but the glucosides in the majority of these have been relatively little investigated. The researches of Guignard have shown that myrosin and the glucoside are localised in different cells in the seed and are only brought together when the cellular structure is destroyed or when a solvent is added. The former is contained in cells free from starch, chlorophyll, fatty material and aleurone grains.

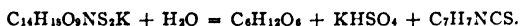
Other enzymes of mustard are sinigrinase (from *Sinapis alba*), which has the same function as myrosin, as well as invertase, amylase, maltase, emulsin and an anaerobic hydrogenase from the seeds of black mustard (Astruc and Mousseron, *Compt. rend.*, 1927, 184, 126).

Several other mustard oil glucosides are known.¹

Mustard is used mainly as a condiment; medicinally its only internal use is as an emetic in poisoning except that white mustard is sometimes employed as a laxative. Externally it is used as an irritant. For local applications the mustard is made up with cold water and rubber solution to a soft uniform cream spread on muslin, cambric or paper and applied directly to the skin; it is kept in contact for 15 to 30 minutes and the skin is then wiped dry. Both black and white mustard are active as sinapisms.

Mustard oil has also been used as a preservative for milk (20 drops/litre), and wine (1 c.c. of a 1% solution in 50% alcohol, per litre). In the former case it is stated to affect only the lactose and protein determinations, and must therefore be removed by extraction with petroleum spirit, or by evaporation, respectively; in the latter, it has the advantages that it is 200 times as effective as sulphur dioxide,

¹ *Glucotropæolin*, $C_{11}H_{19}O_6NS_2K$, occurs in *Tropæolum majus* and *Lepidium sativum*. It is hydrolysed by myrosin to dextrose, potassium hydrogen sulphate and benzyliisothiocyanate.



Gluconasturtium, found in water cress (*Nasturtium officinale*), yields phenylethylisothiocyanate on hydrolysis by myrosin.

Glucoscheirolin, $C_{11}H_{21}O_{11}NS_2K$, H_2O , is an aliphatic sulphone derivative. It occurs in wallflower seeds and is hydrolysed to dextrose and cheirolin.

See also Schneider and others, (*Ber.*, 1912, 45, 2954 et seq.).

and yet does not volatilise or affect the colour or flavour. In white wines however, it has been known to produce turbidity.

The *Sinapis nigra* of the U. S. Pharmacopœia is the dry ripe seed of *Brassica nigra* L. (Koch), of *B. juncea* L. (Cosson) or their varieties, and should contain not more than 5% of other organic material and not less than 0.6% of C_3H_5NCS . The microscopic characteristics are described, and it is stated that removal of a portion of the fixed oil to facilitate grinding is permissible; (as a matter of fact it is often an advantage, as the oil tends to turn rancid). The assay is made on 5 grm. of the powdered material macerated in a stoppered flask with 100 c.c. of water for 2 hours at 37°, and distilled (30 c.c.) into 0.1N silver nitrate solution. The containing flask is stoppered, heated on the following day on the water bath, cooled, made up to 100 c.c. and filtered. Fifty c.c. are then titrated in the manner described for the volatile oil (p. 31).

Commercial Mustard

The mustard of commerce consists of a mixture of the ground seeds of black and white mustard free from hulls, which are incapable of the fine grinding necessary to produce a smooth flour. The manufacturer passes the product through a series of sieves which divide it into pure flour and dressings. The yellow hulls are found in the cheaper grades.

The following analyses made by Presse and Stansell (*Analyst*, 1880, 5, 161) show its composition.

According to the same authors the ash consists mainly of potassium, calcium and magnesium phosphates, with very little chlorides and no carbonates.

In many cases a large proportion of the fixed oil is removed by subjecting the material to hydraulic pressure; during this process thin hard plates called mustard cake are formed and subsequently reduced to fine powder.

Leach ("Food Inspection and Analysis,") quotes in full the analyses of a number of samples made in his laboratory (*J. Amer. Chem. Soc.*, 1904, 26, 1203). The average of all the commercial varieties of flour tested showed: ash 5.03%, ether-soluble 18.6%, reducing matter by acid conversion 6.85%. (See also table of results given in Lunge's "*Chem. Tech. Unt.-Methoden.*")

Constituents, etc.	White mustard					Brown mustard			
	Whole seeds		Farina			Whole seeds	Farina		
	Yorks	Cam-bridge	Super-fine	Fine	Seconds	Cam-bridge	Super-fine	Fine	Seconds
Moisture.....	9.32	8.00	5.78	6.06	8.52	4.35	4.52	5.63
Fixed oil.....	25.56	27.51	37.18	35.74	32.55	25.54	36.96	38.02	36.19
Cellulose.....	10.52	8.87	3.90	4.15	9.34	9.01	3.09	2.06	3.26
Sulphur.....	0.99	0.93	1.33	1.22	1.26	1.28	1.50	1.48	1.30
Nitrogen.....	4.54	4.49	5.05	4.89	4.25	4.38	4.94	5.01	4.31
Total proteins.....	28.37	28.06	31.56	30.56	26.56	26.50	29.81	30.25	26.06
Soluble albumin and myrosin.....	5.24	4.58	7.32	6.67	6.11	5.24	6.46	6.78	6.14
Aqueous extract.....	27.38	26.29	36.31	36.60	33.90	24.22	31.64	32.78	31.41
Volatil. oil.....	0.06	0.08	0.03	0.04	0.03	0.47	1.44	1.50	1.38
Potassium myronate.....	1.69	5.14	5.37	4.94
Total ash.....	4.57	4.70	4.22	4.31	4.30	4.98	5.04	4.84	4.91
Soluble ash.....	0.55	0.75	0.44	0.55	0.33	1.11	1.01	0.98	0.77

To determine sinigrin and sinapin thiocyanate, Gerard and Duprée digest powdered mustard with a mixture of equal parts of water and alcohol in a reflux apparatus. The extract is evaporated and dried at 105° to constant weight, and the residue incinerated so as to transform the acid sulphate into neutral potassium sulphate. The weight of sulphate, multiplied by 4.77, gives the weight of sinigrin, and this deducted from the total weight of the extract gives that of the sinapin thiocyanate. It is preferable, however, to estimate sulphate in the ordinary manner and to calculate from this.

Cruciferous seeds all yield traces of allylthiocyanate or closely allied compounds on treatment with water, but the quantity is very minute, except in the case of black mustard seeds. Thus V. Dircks (*J. Chem. Soc.*, abstr., 1883, 245) obtained from black mustard-seed cake 1.39% of volatile oil; from yellow mustard cake, 0.018; from rape-seed cake, 0.020 to 0.109 (the proportion of oil apparently decreasing with the age of the cake); from rape seeds, 0.018 to 0.037; from turnip seeds, 0.038; and from the seeds of *Sinapis arvensis*, 0.006% of volatile oil. *Brassica juncea* yields Indian mustard oil ("rai") which was found by Sudborough and his co-workers (*J. Indian. Inst. Sci.*, 1926, 9A, 25) to contain the following acids,—Oleic, 32.3; erucic, 41.5; linolic, 18.1; linolenic, 2.7%; but no stearic. The average percentages of allyl mustard oil in various black mustards are: English, 1.39; Greek, 1.20; French, 1.08; Sicilian, 0.99; Italian, 0.99; Bombay, 0.81; Roumanian, 0.4. Mustard-seed

cake is highly irritating to cattle, and mustard should be rigidly excluded from cattle foods. Hence the determination of the mustard oil in seed cakes is sometimes of considerable practical importance.

For the micro-detection of mustard oil the sample is distilled, and concentrated ammonia added to the distillate, which is evaporated to 5 c.c. after 12 hours. A drop evaporated on a microscope slide yields crystals of allylthiocyanate, which appear yellow or brown if 1 drop each of 10% phenylhydrazine and 10% sodium acetate solutions in glycerol are previously added.

For the **estimation of the pungent volatile oil** in cruciferous seeds and seed cakes several methods have been proposed, depending on the separation of the volatile oil from fixed matters by distillation in a current of steam, and its subsequent conversion into some definite and readily-weighed sulphur compound. The plan usually adopted is to mix the crushed seeds or oil-cake with about ten times its weight of cold or slightly warm water, allowing it to stand for a time varying from half an hour to 6 hours, and then volatilising the oil formed by blowing steam through the flask. Various workers, however, insist on the importance of adherence to certain conditions in order to obtain satisfactory results. Dircks recommends that the finely-powdered substance should be mixed with 10 parts of water, and the mixture allowed to stand for 9 hours at 50°, this being necessary to permit the easy distillation of the oil. Steam and air are then blown simultaneously through the mixture, and the distillate collected in alkaline potassium permanganate, which is subsequently treated with hydrochloric acid and the sulphate formed precipitated as barium sulphate.

A later method of Schlicht (*Z. öffent. Chem.*, 1903, **9**, 37), a modification of Dircks' method, is as follows: 25 grm. (or less, if much mustard oil is present) is digested with water for 4 hours at the ordinary temperature, in a flask connected with a condenser and a receiver containing potassium permanganate, as in the foregoing method. It is then boiled in the same apparatus for 15 minutes, and, after cooling, a solution of myrosin is added and allowed to act for 16 hours. The solution is then steam-distilled and the distillate collected in 50 c.c. of a saturated solution of potassium permanganate, to which is added potassium hydroxide equal in weight to one-fourth of the weight of permanganate present. After

150 to 200 c.c. of water have passed over, the distillate is thoroughly shaken and the excess of permanganate reduced by adding pure alcohol (25 c.c. of alcohol reduce 5 gm. KMnO_4), the whole diluted to a known volume and, after filtering through a dry filter-paper, an aliquot portion (preferably one-half) is used for the determination of sulphate in the ordinary way. In order to oxidise any sulphite that may have been formed by the reducing action of aldehyde, it is necessary to add a little iodine to the measured portion of the solution after acidifying with hydrochloric acid, to heat gradually to boiling point, and then to precipitate with barium chloride. (BaSO_4 found $\times 0.4249$ = mustard oil.) Instead of using myrosin, an alternative method is to digest 25 gm. of the mustard flour with 300 c.c. of water containing 0.5 gm. of tartaric acid for 16 hours at the ordinary temperature, and then to distil with steam.

Roeser's method for the estimation of mustard oil in mustard flour (*Analyst*, 1902, **27**, 197) is as follows: 5 gm. of the sample with 60 c.c. of water and 15 c.c. of 60% alcohol are set aside for 2 hours. The oil formed is distilled into a flask containing 10 c.c. of ammonia, about two-thirds of the liquid being distilled over. 10 c.c. of 0.1*N* silver nitrate are added, and the mixture allowed to stand for 24 hours. The solution is made up to 100 c.c., filtered, and 50 c.c. treated with 5 c.c. of 0.1*N* potassium cyanide solution. The excess of this is titrated with silver nitrate solution, potassium iodide (5%), made slightly ammoniacal, being used as indicator. The number of c.c. of silver nitrate taken up by the oil, multiplied by 0.6274, gives the percentage of mustard oil present.

The French Codex method has been modified by Raquet (*J. Pharm. Chim.*, 1920, **22**, 92), and by Luce and Doucet (*id.*, 1922, **25**, 458). The former carries out the maceration with alcohol for 1 hour at 30–35°, and as he then obtains results 20–38% higher, he recommends that the French standard should be raised from 0.7 to 0.9%. The latter authors macerate with water for 1 hour independently of the temperature, and precipitate the sulphide by ammoniacal silver nitrate acting for 6 hours in the cold, or for 1 hour at 80–85°, under a reflux condenser. Prolonged maceration gives low values, as the result of a secondary reaction which destroys the oil, but which is negligible during the first hour. They also obtain higher results and suggest 1% of oil as the standard value for a good quality mustard flour.

Colombier (*Ann. Falsif.*, 1926, **19**, 160) has introduced a further modification into this method, in that he macerates 5 grms. with water, and adds 25 c.c. of alcohol or sodium fluoride after 1 hour, if a longer time is necessary. He distils 90 c.c. (instead of 65 c.c.), and after the precipitation of the silver sulphide, titrates the excess of silver nitrate reagent with 0.1*N* potassium thiocyanate solution. If the presence of other substances which may precipitate silver nitrate is suspected, Jörgensen's method (*ibid.*, 1909, **2**, 372) is very useful. The thiosinamine precipitate is collected in ammonia, filtered, washed and weighed, and the nitrogen content (18–24%) determined by Kjeldahl's method.

A method, which has at any rate the advantage of rapidity, is described by Morvillez and Meesemaeker (*J. Pharm. Chim.*, 1924, **30**, 236), who allow a known excess of 0.1*N* iodine solution to react with the distillate (acidified with sulphuric acid) in the dark for 15 minutes, add chloroform, and titrate the excess of iodine with a solution of sodium thiosulphate.

Astic and Mousseron (*J. Pharm. Chim.*, 1927, **119**, 313) obtain complete liberation of the oil by raising the temperature of maceration slowly to 70°, whilst, in order to avoid oxidation of the silver sulphide precipitate, it has been suggested recently that 50 c.c. of the distillate should be heated under reflux for 30 minutes with 20 c.c. of a 0.5*N* alcoholic solution of potassium hydroxide, the excess of which is titrated. Alternatively, the dextrose may be determined on the filtrate and washings from the maceration process after defecation with neutral lead acetate, the excess of which is subsequently removed by ammonium carbonate.

Volatile oil of mustard, *Allylthiocyanate*, C_3H_5NCS , is prepared by distillation of the seeds of black mustard after the fixed oil has been expressed and the ground seeds macerated with tepid water for some hours, so as to allow the myrosin to hydrolyse the glucoside present.

It is a colourless, mobile liquid with an intensely pungent odour and acid taste. It is soluble in water (1 in 50), in 70% alcohol (1 in 10), and in organic solvents. Sp. gr., 1.014 to 1.025; b. p. 148 to 156°; refractive index, 1.525–1.535. On distillation, the first and last fractions should have the same sp. gr.; this eliminates most adulterants. Delage (*Ann. Falsif.*, 1924, **17**, 336) states that 7% should distil at 148.5–149.9°, 96–97.7% at 150–150.5° (C_3H_5NCS),

and 1-2% remains in the residue. The crude oil consists almost entirely (95-99%) of allylthiocyanate with small quantities of allyl cyanide, carbon disulphide and sulphur compounds, such as $C_3H_5.NH.NCS.SO_2OK$. It should give no coloration with ferric chloride, indicating absence of phenols.

It forms a solid non-volatile compound with ammonia which may be used for its estimation (see Vol. IV, p. 513). On adding excess of ammonia and alcohol to mustard oil the odour disappears and rhombic crystals of thiosinamine or allylthiocarbamide are formed, m. p. 74° . Thiosinamine, $CS.N_2H_3(C_3H_5)$, is readily soluble in water, alcohol and ether, and has a faint leek-like odour.

Volatile oil of mustard is an extremely powerful irritant and causes very rapid vesication when applied to the skin.

The oil is also made artificially by the distillation of allyl iodide and potassium thiocyanate from alcoholic solution; heat converts the allylthiocyanate first formed into mustard oil. The allylthiocyanate content of mustard oil increases during the first months after its preparation, and then decreases. Both the natural and synthetic oils become discoloured in time, and deposit a dark orange or yellow precipitate. From this, a partly soluble portion was found to consist of allylamine and ammonium sulphates, whilst the insoluble residue contained chiefly ψ -thiocyanogen ($C_3HN_3S_3$).

The British Pharmacopœia describes the oil (*Oleum Sinapis Volatile*) as the pale yellow or colourless distillate obtained from the defatted seeds of black mustard macerated with water for several hours. The oil (0.1 grm. in 5.0 c.c. of 90% alcohol) is precipitated with 30 c.c. of 0.1 *N* silver nitrate solution and 3 c.c. of ammonia, heated at 80° for 30 minutes, well shaken, made up to 100 c.c. at 15.5° , and filtered. To 50 c.c. of filtrate and 4 c.c. of nitric acid are added a few drops of ferric sulphate and enough 0.1 *N* ammonium thiocyanate solution to produce a permanent red colour. The maximum titration should be 5.7 c.c., corresponding with not less than 92 grm. of C_3H_5NCS in 100 c.c. of oil.

The following are the requirements of the United States Pharmacopœia X:

A volatile oil obtained from black mustard (freed from its fatty oil) by maceration with water and subsequent distillation, yielding, when assayed by the process given below, not less than 93% of allylthiocyanate. The label should state whether natural or synthetic.

A colourless or pale yellow, limpid, and optically inactive liquid, having a very pungent and acrid odour. *Great caution should be exercised when smelling this oil*; it should not be tasted without being highly diluted. Sp. gr., 1.013 to 1.020 at 25°; refractive index, 1.5268–1.5280 (at 20°). Miscible with alcohol in all proportions, forming a clear solution. It is kept in the dark in amber stoppered bottles.

If to 3 gm. of the oil 6 gm. of sulphuric acid are gradually added, the liquid being kept cool, the mixture, upon subsequent agitation, will evolve sulphur dioxide, but it will remain light yellow in colour, and although at first clear, it will afterwards become thick and occasionally crystalline, and the pungent odour of the oil will disappear.

If a portion of the oil is heated in a flask connected with a well-cooled condenser, it should distil completely between 148 and 154°, and both the first and the last 10% fractions of the distillate should have the same sp. gr. as the original oil (absence of *alcohol, chloroform, petroleum hydrocarbons, fatty oils*, or more than traces of *carbon disulphide*).

If a small portion of the oil is diluted with 5 times its volume of alcohol, the solution should be neutral to litmus, and when a drop of ferric chloride test solution is added, no blue or violet colour should be produced (absence of *phenols*).

Assay.—Five c.c. of a solution of 4 c.c. of oil in 100 c.c. of alcohol are heated under a reflux condenser with 50 c.c. of a 0.1*N* silver nitrate solution and 5 c.c. of ammonia for 1 hour, cooled and made up to 100 c.c. with water. The filtrate (50 c.c.) with 5 c.c. of nitric acid and 2 c.c. of ferric ammonium sulphate is titrated with 0.1*N* potassium thiocyanate solution till a permanent red colour is obtained; 1 c.c. is equivalent to 0.004957 gm. of C_3H_5NCS . The A. O. A. C. (*loc. cit.* p. 15) recommends a similar method.

The oil from the seeds of *Brassica campestris chinoleifera* has been used as an adulteration for mustard oil.

Adulterations of Mustard

Mustard is frequently found adulterated, the most common additions being cayenne pepper (to contribute pungency), mustard hulls, wheat flour, turmeric, millet and other weeds, seeds, rice, potato and corn starch. Mineral adulterations are readily detected by a determination of the ash. The United States standard is

8%. On the other hand, a low ash may indicate admixture with cereals. Wynter Blyth states that the total ash averages 5%, and that if below 4 or above 5.5%, the mustard is adulterated.

The addition of finely ground hulls to mustard is said to be a common practice, and it is difficult to make any restriction as to the amount which should be present. Microscopic examination is the best means of identification, and samples in which hulls predominate over the cellular tissue are certainly adulterated. Hulls are characterised by a cellular epidermal layer, with mucilage, and sub-epidermal cells with thick angles (white mustard). The palisade cells are yellow and reticulated in black, but not in white or brown mustards. The microscope is also effective in determining adulteration with starches or such substances as wheat bran. Mustard may sometimes naturally contain a little starch, but this amounts at the most to 0.1%.¹ (See Collin, *Ann. Falsif.*, 1909, 206.)

The detection of wheat or other cereal flour in mustard presents no difficulty, but the quantitative determination is not easy. By exhausting with ether and alcohol, the fixed oil and glucosides may be removed, and starch estimated in the residue by any of the ordinary methods. Although mustard hulls have been found to give some reducing matter after treatment with diastase, some workers consider that the direct estimation of starch by the diastase method gives a much more reliable figure than estimations based either on the acid inversion or on the proportion of fixed oil. Since the fixed oil absorbs iodine, the sample should be first boiled with a solution of potassium iodide when the starch is being examined microscopically by means of the iodine stain, and comparison made with genuine mustards to which known amounts of flour have been added.

Estimations based on the proportion of fixed oil were formerly more common. The usual mixtures of black and white seed yield about 35% of fixed oil, so that the percentage of fixed oil as determined by extraction $\times 2.857$ will give the amount of real mustard flour present.

This method is invalidated if any portion of the fixed oil has been removed during manufacture, and the results will be falsified if oil has been added together with the cereal. The lowering in the percentage of nitrogen in the water and fat-free sample may also be calculated.

¹ By the alcoholic potash method from 1 to 2% of something resembling starch is often found.

Adulteration of mustard seeds by brans may also be indicated by the ratios of the constituents. In the following ranges of values, the figures in brackets are those obtained from the brans, and it will be seen that the two sets vary widely:— N/fibre 0.5–1.0 (0.09–0.42); P_2O_5/fibre 0.28–0.49 (0.01–0.17). MgO/fibre 0.07–0.16 (0.01–0.06); CaO/MgO 0.79–1.71 (1.2–7.2); $CaO \times \text{fibre}/P_2O_5 \times N$ 0.26–0.78 (1.3–52.3); $CaO \times \text{fibre}/P_2O_5 \times N \times MgO$ 0.21–0.88 (1.6–19.66).

Blyth states mustard contains from 33.9 to 36.7% of oil,¹ wheat-flour from 1.2 to 2.1%, and takes the mean value given by the two

$$32.7x + 120 = 100y$$

$$34.7x + 200 = 100y$$

equations where $x = \%$ of mustard in the mixture, $y = \%$ of oil found.

Determination of the sulphur, which in genuine mustard flour averages about 1.4%, enables any gross adulteration with cereal to be checked. This is conveniently estimated by means of alkaline permanganate. The mustard flour is boiled with excess of alkaline permanganate, and the sulphate formed precipitated by barium chloride. The method of determination by calculation from the sulphur is not to be encouraged.

The cheaper grades of mustard flour made from the wild mustard which grows in the wheat fields contain small amounts of wheat and the seeds of various weeds. The commonest of the wild mustards is Dakota mustard or charlock (*Brassica sinapistrum*), a common weed in corn fields. It contains a dark brown substance in the palisade cells, which on treatment under the microscope with chloral hydrate and gentle heating becomes blood red. Its presence in mustard flour can thus be readily detected.

The addition of wheat flour to mustard is based on the old idea that it is necessary as a preservative and to prevent lumping. In particular, the presence of some 1% of flour is claimed to be necessary for mustard which has to stand variations of climate, and a certain amount of starch contamination may also occur during the harvesting.

¹ F. Sutton states that the amount of fixed oil in the seeds varies according to the soil, climate and locality. Variations from 30 to 47% in the farina of black seeds, and of 23 to 38% in white seeds, have been observed, but as a rule manufacturers of repute avoid the use of the commoner kinds, which are bad in colour and flavour and are used for making mustard cake for manure.

Under the Sale of Foods and Drugs Act the addition is permitted only if duly announced by label, such mixed articles being commonly known as mustard condiment. Pepper is detected from the taste of the residue obtained from the evaporation on the water bath of an alcoholic extract of the mustard, and from the acid fumes evolved when it is heated.

The U. S. Department of Agriculture (Food Inspection Decision, 192, June 1923) has issued the following standards. *Ground Mustard Seed* (Mustard meal)—unbolted, ground mustard seed. *Mustard Cake*—ground mustard seed with a portion of the fixed oil removed. *Mustard Flour* (Ground Mustard) a powder made from the seed, with only a few hulls and a portion of the oil, and containing not more than 1.5% of starch and not more than 6% of ash. *Prepared Mustard*—a paste of the ground seed and/or flour, and/or cake, with salt, and vinegar, with or without sugar (sucrose) spices or other condiments. The fat-, sugar- and salt-free solids contain not more than 24% of carbohydrates (calculated as starch), not more than 12% of crude fibre and not less than 5–6% of nitrogen.

The A. O. A. C. (*loc. cit.* p. 15) methods of examining prepared mustard (official, except where otherwise stated) are included in the following:—

Prepared Mustard

1. **Preparation of Sample.**—The solid portion of the material is commonly in a finely divided condition and does not require grinding, but as a suspension tends to settle, leaving a more or less clear liquid on the surface, thorough mixing is absolutely essential. This may be accomplished by stirring well immediately before removing each portion for analysis.

2. **Solids.**—Dry 5 gm., evaporated with a little water in a flat-bottomed platinum dish on the water-bath, to constant weight, in an oven at 100°.

3. **Ash.**—Char the dry residue, obtained in the determination of moisture, at as low a temperature as possible, extract the charred mass with hot water, re-ash the paper and residue, and weigh it with the residue from the evaporated extract.

4. **Salt.**—Determine chlorine in the ash either gravimetrically or volumetrically. For this purpose the ash is prepared after the

addition of sodium carbonate to the sample, and finally dissolved in nitric acid.

5. Protein.—Determine the nitrogen in 5 gramm. by the Kjeldahl method (see Vol. I) and multiply the result by 6.25.

6. Acidity.—Weigh 10 gramm. into a 200 c.c. graduated flask, make up to the mark with water, shake, filter through a dry paper, and determine the acidity in 100 c.c. by titration with 0.1*N* alkali, using phenolphthalein as indicator. State the results as acetic acid. 1 c.c. of 0.1*N* alkali is equivalent to 0.0060 gramm. of acetic acid.

7. Copper-reducing Substances Calculated as Starch.—Proceed as directed under the A. O. A. C. Method for the determination of starch by acid hydrolysis (Vol. I, page 530), except that 10 gramm. of the material are treated directly with 200 c.c. of water and 20 c.c. of hydrochloric acid (sp. gr. 1.125), without previous washing or extraction, cooled, heated under reflux for 2.5 hours, almost neutralised with sodium hydroxide, and the solution made up to 250 c.c. before filtering and drawing off the aliquot part. The amount of dextrose is multiplied by 0.90.

8. Crude Fibre, Tentative.—Shake 10 gramm. with 50 c.c. of 95% alcohol, add 40 c.c. of ether, and centrifuge after 5 minutes. Decant the liquid and repeat the operation twice with 40 c.c. portions of ether. The residual ether is evaporated, and the usual fibre determination made (Vol. I, p. 72). Care should be taken to avoid "balling," and the isolated fibre should be washed with alcohol and ether before it is weighed.

9. Ether Extract (Tentative).—Mix 10 gramm. with 30 gramm. of sand, heat on the water-bath and in the steam-oven till dry, grind and extract in a Soxhlet apparatus with ether (sp. gr. 0.720) and dry the residue, after evaporation of the solvent at 100°, till constant in weight.

10. Detection of Preservatives.—Proceed as directed in Vol. IX.

11. Detection of Colouring Matter.—Proceed as directed in Vol. V, page 425 *et seq.* Turmeric was at one time largely used to conceal the paleness of tint caused by the addition of flour or other diluent. It may be detected by the microscope or by the boric acid test after extraction with methylated spirit. Nitro-dyes, in particular dinitro- α -naphthol (Martius' Yellow), Naphthol Yellow S and oil-soluble azo-dyes are also used.

The latter are detected in the same manner as butter colours (see Vol. V, p. 435). Leach draws attention to samples of mustard in which the colour is due largely to the presence of the deep yellow fixed oil which has been restored to the powder after pounding. The yellow colour of genuine mustard is water-soluble.

French Mustard.—Dijon mustard is prepared by grinding the seeds moistened with verjuice made from vinegar or white wine and vinegar, the paste being forced through sieves. 100 kgrms. of seeds yield about 300 kgrms. of mustard and 30 kgrms. of husk.

Carles (*Ann. Falsif.*, 1918, **II**, 310) gives the following analyses: Water 62–75%, ash 2.5–11.3%, soluble ash 1–9.5%, sodium chloride 0.66–7.25%, K_2O 0.08–0.25%, allyl mustard oil 0.056–0.257%, acidity 1.8–4.8%, acetic acid 0.5–3.5%, tartaric acid 0.84%, fat 10.5%. Eight mustards of the first and second quality contained 0.313, 0.301, 0.288, 0.240, 0.283, 0.323, 0.288, 0.430% of volatile oil of mustard, respectively. The amount of acetic acid in these varied from 1.5 to 0.16%; it is usually below 0.8%.

According to the resolution of the manufacturers concerned, only the seeds of *Brassica nigra* may be used for Dijon mustard. Other Cruciferæ are frequently substituted. According to Carles, seeds gathered in 1907 showed the following amounts of volatile oil (essence):

<i>Brassica nigra</i> ,	0.9 to 1.3%	
<i>Brassica juncea</i> ,	0.85%	
<i>Sinapis glauca</i> ,	0.62%	
<i>Sinapis dichotoma</i> ,	0.38%	
<i>Brassica rapa</i> ,	0.54%	
<i>Brassica napus</i> ,	0.23%	(rape)
<i>Sinapis arvensis</i> ,	0.11%	(charlock)
<i>Eruca sativa</i> ,	0.56%	

The recognition of the whole seeds is relatively simple, but in paste-form identification is almost impossible. However, as a rule, the cheaper qualities are less finely ground.

Glucosides of Digitalis

The leaves and seeds of the purple foxglove (*Digitalis purpurea*) contain active principles, many of which are glucosides. The leaves of the oleander also contain 2 crystalline glucosides of a

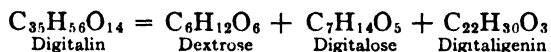
similar character. The chemical knowledge of digitalis is still far from satisfactory, but the glucosides *digitalin*, *digitoxin*, *digitonin*, *gitalin*, *gitonin* and *gitin* have been characterised with some certainty; the nature of *gitoxin* is still uncertain. These glucosides are unstable, and it is probable that many of the preparations described by various workers in this field really represent mixtures.

The leaves contain digitalin and digitoxin, whilst digitalin and digitonin are present in the seeds and represent 5% of the dry matter. The seed glucosides disappear during germination and are stored in the leaves in a slightly different form, where they remain constant in amount. Digitonin is a member of the saponin group and possesses all the properties of these substances. It appears to aid the solution of some of the other glucosides. Digitoxin possesses the characteristic action of digitalis preparations on the heart, and is the most toxic; digitalin has a similar but weaker action.

Digitalin has the composition $C_{37}H_{58}O_{14}$ or $C_{35}H_{56}O_{14}$, according to its origin; it is a colourless amorphous powder, m. p. 229° , very sparingly soluble in water (1:1,000), readily soluble in hot alcohol, and when pure crystallises from the hot saturated solution, on cooling, in characteristic granular masses. The presence of the other digitalis glucosides prevents this characteristic separation. It crystallises in needles from 85% methyl alcohol, and is sparingly soluble in ether or chloroform.

Concentrated sulphuric acid dissolves it with a golden yellow coloration; this changes to a magnificent rose or violet-red on the cautious addition of potassium hypobromite or other oxidising agent. Thus, with ferric chloride and sulphuric acid the colour is at first an intense golden yellow, then red and changes to a permanent reddish-violet. It is advisable to use only very little digitalin.

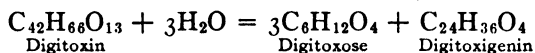
It is best hydrolysed by alcoholic hydrogen chloride to avoid the formation of resin, and forms dextrose, a sugar, $C_6H_{12}O_6$, termed by Kiliani *digitalose*, and *digitaligenin*, $C_{22}H_{30}O_3$, or $C_{23}H_{32}O_3$. It was thus shown to be a tri-hydroxy lactone, with one free hydroxyl group and the others attached to the digitalose and digitaligenin, respectively.



Digitaligenin crystallises from 90% alcohol in granular aggregates of colourless needles, m. p. 210 to 212° .

Digitoxin, $C_{42}H_{66}O_{13}$, the more active principle of *Digitalis* crystallises from a mixture of methyl alcohol and chloroform in slender anhydrous prisms which melt incipiently at 240° , or at 145 – 150° when produced in hydrated crystals from dilute alcohol. It is insoluble in hot or cold water, soluble in alcohol, chloroform or ether, and may thus be separated from its allied glucosides. (Windaus and Freese, *Ber.*, 1925, 58, [B], 2503). It gives a characteristic colour reaction when it is dissolved in glacial acetic acid and concentrated sulphuric acid containing a drop of ferric chloride is cautiously added. A dirty brown or bluish-green band appears at the zone of contact, changing to an intense indigo-blue band. A greenish or brownish-green coloration is obtained on heating with concentrated hydrochloric acid.

It is hydrolysed, even at the ordinary temperature, by alcoholic hydrogen chloride to digitoxose and digitoxigenin¹



Digitoxose crystallises in prisms or plates, m. p. 102° , and is dextrorotatory. Kiliani has shown it to have the constitution $CH_3.CH(OH).CH(OH).CH(OH).CH_2.CHO$.

Digitoxigenin forms colourless crystals, m. p. 230° ; alcoholic sodium hydroxide converts it into salts of an acid, $C_{22}H_{34}O_5$, crystallising in bunches of needles, m. p. 220 to 230° . By the action of strong hydrochloric acid *anhydrodigitoxigenin*, $C_{22}H_{30}O_3$, is formed; it crystallises in prisms, m. p. 215 to 220° .

Digitonin has the composition $C_{55}H_{90}O_{29}$. It comprises 50% of the mixed digitalis glucosides and belongs to the saponins. It softens at 225° and melts completely at 235° . The crystals dissolve sparingly in cold water, more readily in hot, to give a frothy opalescent solution. On evaporation, the solution yields only a gummy mass, all attempts to crystallise digitonin from water being hitherto unsuccessful. The aqueous solution is laevorotatory ($[\alpha]_D = -49.25^\circ$), and is precipitated by tannin, ammoniacal lead acetate, and baryta water. Digitonin is only slightly soluble in absolute alcohol, and still less so in ether, chloroform, or petroleum spirit. It is almost completely precipitated by adding ether to its alcoholic or aqueous solution, and may then be separated from the

¹ The recently published work of Windaus (see *Bureau Chem. Abs.*, 1928, A, 276) gives the formulæ of digitoxin and digitoxigenin as $C_{41}H_{64}O_{12}$ and $C_{22}H_{34}O_4$, respectively.

saponins. The commercial product contains 70–80% of glucoside, with 10–20% gitonin and 5–15% of other glucosides. It crystallises in slender needles from 85% alcohol.

When amyl alcohol is added to an aqueous solution of digitonin, the glucoside is rapidly separated in a crystalline form. If a hot mixture of amyl and ethyl alcohols is used, the solution deposits, on cooling, long nacreous laminæ, which contain amyl alcohol and water of crystallisation. Similar crystalline compounds of digitonin with other alcohols and with phenol have been described by J. Houdas, (see Kiliani, *Pharm. J.*, [iii], **24**, 45.)

Digitonin dissolves in strong sulphuric acid, with red colour, an addition of a drop of bromine water intensifying the colour, but not changing it to violet. Sulphuric acid diluted with its own volume of water produces a yellowish coloration in the cold, changing to red and finally to black on heating. Concentrated hydrochloric acid gives a colourless solution which, after a time or on heating, turns yellow and then violet-red, with a slight greenish fluorescence.

It combines with a molecule of cholesterol in alcoholic solution to give a crystalline precipitate. This reaction affords a valuable test for digitonin and a method for its separation from the commercial digitalin (Windaus, *Ber.*, 1909, **42**, 238). It is hydrolysed on heating with alcoholic hydrogen chloride to a hexose (4 mols.), a pentose and digitogenin, $C_{26}H_{42}O_5$, which crystallises in very slender needles, softening at 240° , and it forms a number of crystalline additive compounds (Windaus and Weinhold, *Z. physiol. Chem.*, 1923, **126**, 299). Reichard (*Pharm. Zentr.*, 1913, **54**, 217) has described a number of reactions of digitonin. A drop of cobalt nitrate solution is evaporated, and digitonin and a drop of glacial acetic acid added. After 30 hours in the air a mass of red crystals results. The digitonide of oxycholesterol, which occurs in animal fats associated with cholesterol, forms rhombic plates, m. p. 215° .

Gitalin (ψ -digitoxin), $C_{28}H_{48}O_{10}$, is also prepared from *D. purpurea* by extraction of an aqueous extract of the plant with amyl or butyl alcohol. The solution, concentrated under reduced pressure, is precipitated with ether, the gitalin extracted with chloroform, and reprecipitated with ether (U. S. P. 1925, **1**, 586, 116). When a solution of digitoxin, gitalin and anhydrogitalin in a mixture of equal volumes of chloroform and methyl alcohol is treated with ether, gitalin remains in solution and the two other constituents are

precipitated, (Kiliani, *Arch. Pharm.*, 1913, 251, 562). Gitonin and digitonin form crystalline compounds with amyl alcohol, and this property is used as a means of separation.

It is amorphous, neutral, almost insoluble in water, and physiologically active, and evaporation of a solution in alcohol yields anhydrogitalin, $C_{26}H_{46}O_9$. Both compounds are hydrolysed to digitoxose and anhydrogitaligenin, $C_{22}H_{34}O_5$. Kiliani (*loc. cit.*) states that it is a mixture of glucosides; $[\alpha]_D^{15} - 25.2$ (in chloroform, -18.8 (in alcohol), m. p. 155°). Cloetta (*Arch. exp. path. Pharm.*, 1926, 112, 261) also identified *i-gitalin*, $C_{17}H_{28}O_6$, m. p. 245° , and *bigitalin*, $C_{40}H_{64}O_{14}$, m. p. 282° .

Gitoxin, $C_{42}H_{66}O_{14}$, is hydrolysed to digitoxose and gitoxigenin, $C_{24}H_{36}O_5$. *Digitan*, $C_{35}H_{50}O_{10}$, has the same pharmacological effect, but only 75% of the activity of digitoxin. It gives Keller's reaction. The commercial product is often diluted with lactose.

Oleandrin, $C_{31}H_{48}O_9$, occurs in oleander leaves, the activity of which is 2.5 times that of digitalis. According to Tauber and Zelener (*Arch. Pharm.*, 1926, 264, 608) it is a mixture of the compounds $C_{24}H_{34}O_7$ (m. p. 230°), $C_{33}H_{47}O_8$ (m. p. 224°), and another glucoside (m. p. $98-120^\circ$). The first is separable by its insolubility in a mixture of ether and alcohol.

Cymarín, $C_{30}H_{44}O_9$, m. p. 139° , from Canadian hemp (*Apocyanum cannabinum*) is related to strophanthin in that it gives the same hydrolysis product (cymarigenin or strophanthidin, $C_{23}H_{30}O_5$), together with cymarose $C_7H_{14}O_4$ (digitoxose methyl ether).

Gitonin, $C_{49}H_{80}O_{28}$, m. p. 272° , $[\alpha]_D - 50.7$ (in pyridine), was obtained by Windaus from Merck's preparation of digitonin (*Digitalinum germanicum*); it is hydrolysed to galactose (3 mols.), a pentose and gitogenin, $C_{26}H_{42}O_4$, m. p. 272° (diacetate m. p. 243°). Reichard's test (p. 39) gives a green residue.

Physiological Activity and Assay of Digitalis.—According to Hirohasi (*Chem. and Drug.*, 1913, 82, 18) digitalis leaves gathered from different parts of the plant differ in their physiological effect, there being a diminution in the latter from the top downwards. The leaves are best collected before inflorescence. The flowers have a maximum of activity during budding, and there is no difference in activity between red and white flowers. An infusion of the leaves can be evaporated without loss of any of its physiological effects, and its keeping properties are thereby improved. The

more active constituents are chloroform-soluble, and an aqueous extract is more active than an alcoholic extract. Wijngaarden (*Arch. exp. path. Chem.*, 1926, **114**, 21), however, prepared a highly active extract by the use of hot absolute alcohol in a Soxhlet apparatus, the leaves being dried at 15–30° and stored for a period. Hintzle-mann (*id.*, 1926, **112**, 56) found that the presence of alkali accelerated the rate of deterioration of digitalis extracts, which must therefore be analysed as rapidly as possible, and without the addition of acid or alkali. In long-period tests 0.3% of phenol should be added. Hatcher (*Amer. J. Pharm.*, 1914, **86**, 567) states that digitalis of the first year's growth is probably as active as that of the second, and the cultivated is as active as the wild-grown plant. When properly dried and stored, digitalis will keep indefinitely.

The physiological activity of various samples of digitalis varies enormously according to the proportion of the constituents, and the so-called active principles extracted from the leaf vary even more than the crude preparations. Further, the total amount of glucoside present is of no value as an indication of its activity. The only trustworthy method of standardisation of these and similar drugs is to measure their effects on animals.

The dried leaves of *Digitalis purpurea*, collected just before flowering occurs, are official in the British and U. S. Pharmacopœias. The latter requires the presence of not more than 2% of browned leaves, stems, flowers or other foreign organic matter, and not more than 5% of acid-insoluble ash. The assay is made physiologically by the injection of a tincture through the floor of the mouth into the ventral lymph sac of a frog (*Rana pipiens*, Schreber, 20–30 grm. in weight). The tincture contains not more than 20% of alcohol, and 0.015 c.c. is injected per grm. of body-weight. After 58 minutes at 20° the ventricle should be in systolic standstill, and the auricles dilated. A report of the Pharmaceutical Society of Great Britain states that the use of cats in place of frogs (Magnus's modification of Hatcher's method—*Amer. J. Pharm.*, 1910, **82**, 360) is to be preferred. The dilute tincture is injected (1 c.c. per minute) into the femoral vein of one side of each of 5 cats, artificially respirationed with a mixture of air and ether vapour. The amount (in c.c. per kgrm. of body-weight) needed to cause the blood pressure, as registered by the carotid artery, to drop sharply to zero, (*i. e.*, to cause heart stoppage in systolic contracture), is measured, and is 15.96 for the standard sample of

digitalis prepared according to the International Conference of the Health Section of the League of Nations (1925) and placed at the disposal of the Medical Research Council (*loc. cit.* p. 49). A tolerance of activity of $\pm 25\%$ is recommended.¹ Wible (*Amer. J. Pharm.*, 1926, **98**, 396) confirmed the use of this method and showed that it gives results in agreement with the U. S. P. method. The various physiological methods are also discussed by Lenz (*J. Pharm. exp. Ther.*, 1926, **29**, 407).

An infusion and a tincture of digitalis are used in medicine. The infusion contains some digitalin and digitoxin in suspension, though these are insoluble in water, the emulsification being brought about by the digitonin. In old preparations the glucosides decompose, forming resin-like substances which are very toxic; the ordinary hydrolysis products ("genins"), however, are less active than the glucosides.

Digitalis is a cardiac stimulant; it slows the rate of the beat. Externally it is intensely irritant. Given by the mouth it causes vomiting and diarrhœa.

Commercial Digitalin.—The leaves and powder should be dried and kept in sealed vessels; otherwise the activity is rapidly lost. Crushed or powdered leaves are occasionally adulterated with mullein leaves (*Verbascum thapsus*) which are woolly and have branched hairs; primrose leaves which are spatulate and have straight lateral veins; comfrey leaves (*Symphytum officinale*); matico leaves (*Piper angustifolium*), etc.

Digitalinum germanicum (Merck) consists chiefly of digitonin, gitonin and digitalin; it is a yellowish-white amorphous powder soluble in water and alcohol, insoluble in ether and chloroform.

Homolle's amorphous digitalin is amorphous, sparingly soluble in water, readily soluble in alcohol and chloroform. It consists of digitalin with some digitoxin.

Nativelle's crystallised digitalin is mainly digitoxin; it forms fine white needles bitter in taste, insoluble in water, ether or benzene, and, freely soluble in chloroform.

Other commercial preparations are *digalen* and *digipan*, both of which are aqueous extracts of the leaves and contain gitalin. *Digipuratum* and *digitalysatum* are hot-water infusions of the leaves.

Keller's method of estimating the digitalis glucosides is that most usually employed. The leaves (20 grm.) are extracted with 70%

¹ See addendum, p. 76.

alcohol,¹ the alcohol evaporated on the water-bath to about 25 grm., and the residue dissolved in water, made up to a weight of 222 grm., and purified with lead acetate (25 grm.). The bulky precipitate is poured on to an 18 cm. filter-paper, and from 132 grm. of the filtrate excess of lead is removed by sodium sulphate (5 grm. sodium sulphate in 7 grm. water). The precipitation is carried out in an Erlenmeyer flask which is shaken and placed in a sloping position so that the precipitate settles in the angle. After 4 to 5 hours, 130 grm. of the clear liquid (representing 10 grm. of digitalis), can be decanted. This is made alkaline with ammonia (2 c.c. of 10% solution), extracted several times with 30 c.c. portions of chloroform to remove the digitoxin, the chloroform solution filtered, evaporated, and the residue weighed. It contains fatty and colouring matters besides the glucoside, and is purified by solution in a minimum volume of chloroform and precipitation with a mixture of ether (10 c.c.) and light petroleum spirit (70 c.c.). This purification may be repeated, if necessary.

The aqueous solution after extraction with chloroform is made acid and the digitonin precipitated with tannic acid, the tannates are collected, dissolved in 50% alcohol, lead oxide added, and the mixture evaporated to dryness. The residue is extracted with dilute alcohol and the extract evaporated. More tannin and concentrated sulphuric acid are added to the filtrate from the tannic acid precipitation to precipitate the digitalin. (Compare Vanderkleed, *Amer. J. Pharm.*, 1908, 80, 114, and Burmann, *Bull. Soc. Chim.*, 1910, [iv], 7, 973.)

Martindale (*Pharm. J.*, 1912, 35, 745, 778) claims that the following simple method gives an approximate idea whether a tincture of digitalis is up to the physiological test requirements: 10 c.c. of the tincture are mixed with 10 c.c. of water and precipitated with 3 c.c. of 10% neutral lead acetate solution, a little kieselguhr being added. After standing for 15 minutes the precipitate is filtered off and washed. The lead is removed from the filtrate by the addition of 2 c.c. of 10% sodium phosphate solution, and the filtrate is evaporated to dryness after the addition of 0.2 grm. of calcium carbonate. The residue is mixed with sand and extracted five times

¹ Extraction is judged to be complete as follows: 3 to 4 c.c. of the percolate are evaporated to dryness, the residue dissolved in 3 c.c. of water to which 2 drops of dilute hydrochloric acid have been added, the solution is filtered and tested with tannin solution; it should show no appreciable turbidity.

with chloroform, 10 c.c. being used on each occasion. The extract is evaporated, and the residue extracted with warm water on the water-bath, 10 c.c. and 5 c.c. portions being used, and again with the addition of sand. The filtrate is evaporated to dryness and extracted three or four times with chloroform, 5 c.c. each time, the residue being mixed with sand and thoroughly triturated. The chloroform liquors are evaporated, the residue dissolved in 4 c.c. of glacial acetic acid, and 0.1 c.c. of this solution mixed with 1 c.c. of "sulphuric ammonium molybdate solution" in a 5×1 cm. test-tube, and the depth of colour produced after 5 minutes compared with a standard. The coloration indicates the content of combined "active water-soluble glucosides." The claim of Knudson and Dresbach, that a solution of digitalis, decolorised by means of lead acetate, gives a colour with an alkali picrate proportional in intensity to the pharmacological activity has not been substantiated (see Baljet's test, p. 45).

A recent method for the determination of digitonin (Mellanoff, *Amer. J. Pharm.*, 1927, **99**, 390) is stated to give good results. Small quantities of a 1% solution of cholesterol in 90% alcohol are added to a hot solution of digitonin in 90% alcohol, till precipitation of the equimolecular compound is complete. The filtered precipitate is washed with the solvent, the filtrate tested with more cholesterol, and the digitonin-liberated from the complex by means of xylene. If α - or β -naphthol is used instead, the molecular compound contains 8 or 10 mols. of water, respectively, and must be decomposed with benzene.

A number of colour reactions for these glucosides exist (Granier, *J. Pharm. Chim.*, 1908, **27**, 369). They include:

Keller's Reaction.—(a) 100 c.c. glacial acetic acid and 1 c.c. of 5% ferric sulphate. (b) 100 c.c. strong sulphuric and 1 c.c. of 5% ferric sulphate. The glucoside is dissolved in 1 c.c. of (a) in a narrow tube and 2 c.c. of (b) introduced slowly. Digitoxin gives a bluish-black colour at the zone of contact, and after 2 hours the supernatant acetic acid becomes blue. With digitalin a cherry-red colour is at once formed in the upper stratum of the sulphuric acid.

Lafon's Reaction.—(a) Equal volumes of 95% alcohol and strong sulphuric acid. (b) Very dilute aqueous ferric chloride. A crystal of the glucoside is moistened with a drop of (a) and a drop of (b) placed in proximity. On contact, digitoxin gives an intense greenish-

blue coloration but digitalin does not give the reaction. Both these tests can be applied to the evaporated chloroform extract.

Brissemoret-Derrien's Reaction.—(a) 30 c.c. glacial acetic acid mixed with 20 c.c. of 4% oxalic acid which is reduced to glyoxalic acid by treatment with sodium amalgam until neutral. (b) Strong sulphuric acid. The glucoside is dissolved in (a) and 2 c.c. of (b) carefully added. Digitoxin slowly develops a grey or greyish-green tint at the zone of contact. Digitalin gives a cherry-red tint as with Keller's reaction. The glucoside dissolves in (a) with difficulty.

Baljet's Reaction (*Pharm. Weekblad*, 1918, 55, 457).—By virtue of the lactone structure, digitalin gives a deep red, and gitalin, digitoxin and strophanthin an orange-red colour with picric acid and potassium or sodium hydroxide. Peptones, creatinin, acetone and aldoses also give the reaction, but digitonin, arbutin, and amygdalin do not.

Reichard's test is given on p. 39.

For a critical review of analytical methods for digitalis see Focke (*Arch. Pharm.*, 1910, 248, 365), and for its uses in medicine A. R. Cushny, "The Action and uses of Digitalis and its Allies in Medicine," 1925.

Glucosides of Strophanthus

The seeds of *Strophanthus Kombé*, a climbing plant belonging to the order *Apocynaceæ*, indigenous to eastern tropical Africa, contain the glucoside strophanthin. An extract of the seeds is used by the natives for the preparation of the arrow poison called *Kombé, inée* or *onaje*. The seeds are remarkable for the long *awn* of white silky hairs that is attached to them. These awns are usually removed before exportation, and the commercial seeds are of an elongated oval shape, $\frac{1}{2}$ to $\frac{3}{4}$ in. in length and $\frac{1}{6}$ in. in thickness. They break easily and in section show two straight cotyledons; the odour is characteristic and the taste bitter. A transverse section moistened with sulphuric acid shows a fine green colour.

The commercial drug is often mixed with the seeds of other species of strophanthus.

S. hispidus gives smaller brownish seeds which bear scattered hairs: it gives the green coloration with sulphuric acid, see addendum, p. 78.

S. Courmonti seeds are smaller, of a more lanceolate shape, less bitter, contain abundant prismatic crystals of calcium oxalate in the

seed coat, and give a red coloration with concentrated sulphuric acid.

S. Nicholsoni has whitish, woolly seeds and gives a red colour with sulphuric acid.

S. gratus has brown and glabrous seeds which give a red coloration with sulphuric acid and contain the glucoside *ouabain*.

S. emini has greyish-green seeds which contain clusters of calcium oxalate crystals and give a red sulphuric acid coloration.

The genuine seeds are best recognised by their greenish or fawn colour, by the hairs, which are satiny not woolly, by the absence of calcium oxalate from the embryo, and by the green sulphuric acid coloration.

The seeds of *S. Kombé* contain about 2 to 3% of strophanthin, about 25% of fixed oil; other constituents are choline, trigonelline and kombic acid, the ash amounting to about 4%.

To isolate the glucoside, the freshly powdered seeds are extracted first with ether or carbon disulphide to remove the fat, and then with 70% alcohol. The alcohol is distilled from the extract, the residue dissolved in water, and the solution filtered and treated with tannic acid, an excess being avoided. The precipitate is washed, mixed with lead oxide, dried, and extracted with alcohol. Ether is added to this alcoholic solution to precipitate the glucoside.

Brauns and Closson (*Arch. Pharm.*, 1914, 252, 294) remove fat from the powder with petroleum spirit, extract it twice with 70% alcohol, and clarify the extract with lead acetate, the excess of which is removed by means of hydrogen sulphide. The extract is concentrated slowly at 45°, and the strophanthin crystallised from an alcoholic extract, water being added as evaporation proceeds. The compound $C_{40}H_{58}O_{15} \cdot 3H_2O$ separates as neutral, colourless microscopic crystalline plates, m. p. 158–165° (179° anhydrous), and $[\alpha]_D$ 31.6 (in 94% alcohol) and 28.7 (in water). Crystallisation from water produces an amorphous acid form, $C_{40}H_{58}O_{16}$, which softens at 100° (m. p. 165–170°); when dry, it softens at 160° (m. p. 180°) and has $[\alpha]_D$ 20.6 (in water). Another amorphous variety was extracted by means of alcohol from the seeds of *strophanthus*. All three gave the same hydrolysis product, but the crystalline strophanthin had three times the activity of the others.

All varieties of the drug contain varying amounts (up to 0.2%) of the hæmolytic saponin, *strophanthic acid* (Sieburg, *Ber. deut.*

pharm. Ges., 1913, 23, 278), which forms precipitates with salts of the heavy metals and is hydrolysed to dextrose and strophantigenin. Its colour reactions differ from those of strophanthin. The chemical and physiological properties of the commercial product may therefore vary considerably on account of its varying composition.

Cymarín (see *Digitalis* glucosides) is allied to strophanthin. Jacobs and Hoffman (*J. Biol. Chem.*, 1926, 67, 609), on shaking crystalline strophanthin from *S. Kombé* with chloroform and water, obtained in the chloroform layer a substance which appeared identical with cymarín (m. p. 204–205°, $[\alpha]_D^{20}$ 37.8, in chloroform), and was hydrolysed to cymarose. The aqueous layer yielded *Kombé strophanthin-B*, $C_{36}H_{54}O_{14}$, $1.65H_2O$, (m. p. 176°), which was hydrolysed to strophanthidin, a hexose and a disaccharide of cymarose. The mother liquors contained other similar glucosides.

A method suitable for the assay of the glucoside is described by Caesar and Loretz (*Pharm. Zentr.*, 1905, 46, 859). The crushed seeds are extracted for an hour with absolute alcohol, filtered, and an aliquot portion evaporated to dryness on the water-bath. The residue is treated with petroleum spirit to remove the fat, dissolved in boiling water and treated with basic lead acetate solution. The precipitate is collected and well washed, the filtrate freed from lead by means of hydrogen sulphide, and evaporated to dryness, and the residue, which is crude strophanthin, weighed.

According to Lampart and Müller (*Arch. Pharm.*, 1913, 251, 609), who have compared a number of methods, the most satisfactory way of determining strophanthin in strophanthus seeds or tincture, is an extension of the above method, which gives higher values than any other. The full details are as follows:

“Seven grm. of the finely crushed seeds are boiled in a reflux apparatus for an hour with 70.0 grm. of absolute alcohol. When cold, the whole is made up to the original weight with absolute alcohol, and 50.5 grm. filtered into a porcelain basin. The alcohol is evaporated, and the residue washed with petroleum spirit, which is poured through a filter. The insoluble residues in the filter and basin are boiled with 5–8 grm. of water, treated with 5 drops of lead acetate solution and about 0.2 grm. of kieselguhr, well mixed and filtered into a 100 c.c. flask. The insoluble portion is washed till the runnings no longer have a bitter taste. The filtrate is treated with 5 drops of hydrochloric acid and boiled gently for 2 hours, the

volume being kept between 10–20 c.c. by the addition of distilled water. When cold, the liquid is extracted twice with 10 c.c. of chloroform, which is filtered into a weighed flask. The aqueous portion is again boiled for half an hour, cooled and extracted three times with 10 c.c. of chloroform. If the aqueous portion after warming still tastes bitter, the boiling and extraction with chloroform are repeated. The chloroform is distilled off, and the residue dried in a desiccator and weighed. It consists of strophanthidin, 1 part of which corresponds with 2.187 parts of pure strophanthin. For the tincture 51 grm. (equivalent to 5.0 grm. of the seeds) are heated on the water-bath to remove the alcohol, and the residue is taken up with 20 grm. of hot water, and treated with 15 drops of lead acetate solution and 0.2 grm. of kieselguhr. It is then treated by the method described above for the seeds."

Strophanthin, $C_{36}H_{54}O_{15}$, from *S. Kombé* is pale yellow and amorphous, or a colourless crystalline powder (if from *S. gratus*), having an intensely bitter taste and a faintly acid reaction. It melts at 170 to 172° and is remarkable in being dextro-rotatory, $[\alpha_D]$ 29.16. It is readily soluble in water. The green coloration obtained with 96% sulphuric acid is characteristic and sensitive to 0.001 mgrm. If this solution is gently heated, a grey-blue colour indicates that the glucoside is derived from *S. Kombé*, and not from *S. gratus*. When dissolved in warm potassium dichromate solution it deposits yellow crystals when cooled.

The glucoside is hydrolysed by 0.5% hydrochloric acid at 70° to *strophanthidin*, $C_{23}H_{30}O_5$, and *strophantibiose methyl ether*, $C_{13}H_{24}O_{10}$, which latter is in turn further broken down on hydrolysis, yielding rhamnose, mannose and methyl alcohol.

Strophanthidin crystallises in prisms, m. p. 235° (anhydrous), 169° (hydrated), and is a dilactone. The structures of these compounds are discussed by Jacobs and Gustus (*J. Biol. Chem.*, 1927, **74**, 795 *et. seq.*).

Strophanthin shows certain colour reactions. On heating with mineral acids it gives various shades of green, changing to violet or blue. A solution of phenol or of ferrous sulphate and acetic acid in strong sulphuric acid dissolves it with a violet coloration which turns green on the addition of water. The addition of a trace of ferric chloride and a little sulphuric acid to the aqueous solution

produces a red-brown precipitate. It is precipitated by tannic acid, but is soluble in excess.

A very sensitive test is the green to blue colour or precipitate obtained when the crystals are boiled with 3 c.c. of a 0.2% solution of concentrated hydrochloric acid containing 4 drops of ferric chloride per 100 c.c. After dilution to twice the volume and extraction with amyl alcohol, this turns to lilac and eventually to a stable carmine. If free pentoses are suspected, the glucoside should first be extracted with chloroform and an alcoholic extract of the residue used after evaporation.

The British Pharmacopœia tincture and extract of *Strophanthi semina* (Strophanthus seeds) is prepared from the ripe seeds of *S. Kombé*, free from awns, ground to pass a No. 30 sieve and dried at 45°. The latter may be diluted with lactose. The U. S. Pharmacopœia allows the use of *S. Kombé* or *S. hispidus*, the assay being carried out as for digitalis (p. 41). The minimum systolic dose should not exceed 0.00006 c.c. of a 10% tincture, equivalent to 0.000005 grm. of ouabain, for each grm. of body weight of the frog.

The Pharmaceutical Society of Great Britain (see Burn and Trevan, *Pharm. J.*, 1926, 117, 439) have adopted the recommendation of the International Conference at Geneva (1925) of the Health Section of the League of Nations¹ and take ouabain or *Gratus* strophanthin as the biological standard for Strophanthus. The cat method is used here, as for digitalis (p. 41), except that before they are administered to the anæsthetised cat the tinctures are diluted (1 part in 900). Tinctures are considered satisfactory when, as a result of a test on 3 cats, they are shown to have the equivalent of a 0.26–0.44% solution of ouabain, the tincture of strophanthus-Kombé, B. P., being equivalent to a 0.35% solution of the standard ouabain of the U. S. Department of Agriculture.

This standard was set up as a result of the work of Burn and Trevan (*loc. cit.*) which showed that 6 tinctures of Strophanthus B. P. from different sources, gave the values 1 c.c. = 2.88 – 3.85 mg. (mean 3.46 mg.). This is less than half the value required by the U. S. Pharmacopœia (1 c.c. = 8.33 mg.), which is considered impossibly high, and a value of 4.2 mg. is considered as high as can reasonably be expected with any regularity from good strophanthus

¹ (Report C. 532, M. 183, 1925, 111. C. H. 350, Constable, London.)

seeds. The work of Houghton (Parke Davis Research Laboratory, Reprint No. 22, 1912) confirms this (see addendum, p. 78).

Strophanthus is intensely poisonous—eight or nine times more so than *digitalis*. The action on the heart is very similar to that of *digitalis*, but *strophanthus* has less effect on the nervous system. It is a more effective diuretic than *digitalis* and a much more powerful muscle poison. To avoid deterioration, it should be stored away from light, heat or moisture (Pickering, *J. Amer. Pharm. Ass.*, 1928, **17**, 121).

Pseudo-strophanthin, $C_{40}H_{60}O_{16}$, has been found both in *S. Kombé* (?) and *hispidus*. It is a neutral hygroscopic powder, m. p. 179° , and gives a red coloration with concentrated sulphuric acid. It is less easily hydrolysed than *strophanthin*, yielding ψ -*strophanthidin*, $C_{27}H_{37}O_6CH_3$, and a *disaccharide*, $C_{12}H_{22}O_{11}$. ψ -*Strophanthidin* is the methyl ether of a compound containing an oxygen atom less than *strophanthidin*; it has m. p. 195° . ψ -*Strophanthin* is stated to be more active physiologically than *strophanthin*. According to the British Pharmacopœia Codex it is probably identical with *ouabain* obtained from *S. gratus*.

Ouabain, $C_{30}H_{46}O_{12}$, the arrow poison of the Somalis, is contained in the root and wood of *Acokanthera ouabaio*, in *Strophanthus gratus*, and in the latex of *Strophanthus glaber* (Arnaud, *Compt. rend.*, 1888, **107**, 1162; 1898, **126**, 346, 1208, 1280, 1684, 1873). It forms slender transparent rectangular plates, m. p. 185° , after previous softening, and is sparingly soluble in cold, but more readily soluble in warm water, and in 85% alcohol. It is insoluble in ether, chloroform and absolute alcohol. It contains water of crystallisation varying according to the temperature from 1 to 9 molecules. It is hydrolysed by dilute acid to a *methyl pentose*—perhaps *rhamnose*—and a *resin*, $C_{24}H_{36}O_8$, which readily loses 4 molecules of water, forming a red resin, $C_{24}H_{28}O_4$.

On heating with alkali, *ouabaic acid*, $C_{30}H_{48}O_{13}$, results; this is a gummy amorphous solid, decomposing at 235° , giving *rhamnose* on hydrolysis.

The following table compiled from the results of Richaud (*J. Pharm. Chim.*, 1921, **24**, 161) and of Tiffeneau provides means of distinction between *ouabain* and *strophanthin*.

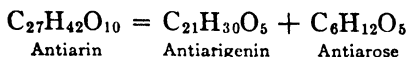
Test	Strophanthin	Ouabain
Solubility.....	1 in 40-43 pts. of water at 15°.	1 in 150.
Solution.....	Yellow, opalescent, bitter and froths.	Clear, colourless, no persistent froth.
Crystalline form.....	Dirty yellow, or white amorphous powder or rosettes of leaflets.	Pure white nacreous powder or small rectangular tablets visible under the microscope.
Hydrolysis reaction. (Warm at 60-70° with 5 c.c. conc. HCl and a crystal of resorcinol)....	Rose colour.	No colour.
Conc. H ₂ SO ₄	Green.	Rose red or yellow brown.
80% H ₂ SO ₄	Green.	No colour.
Conc. H ₂ SO ₄ + phosphomolybdic acid.....	Blue.	No colour.
Conc. H ₂ SO ₄ + tungstic acid....	Green.	No colour.
Conc. H ₂ SO ₄ + vanadic acid....	No colour.	Green.
Conc. H ₂ SO ₄ + phenol.....	Violet.	No colour.
M. p.....	170-172°.	185°
[α] _D	+29.6 (trihydrate).	-24.0 (nonahydrate).

The ouabain used by the U. S. Pharmacopœia as a standard for the assay of strophanthus (*vide supra*) has the formula C₃₀H₄₆O₁₂, 9H₂O. It is crystallised from a mixture of alcohol and ether and becomes anhydrous if heated to 130°, incipient m. p. 180°, [α]_D²⁰ - 31.3 to 31.9. Burn (*loc. cit.*), however, points out that the number of mols. of water of crystallisation varies considerably with the temperature of crystallisation and of drying (e. g. at 30° and 60°, 4 and 3 mols. are present, respectively) and the physiological activity of a given weight of ouabain will depend on its moisture content. This must be stated when the drug is required as a standard. Thus, a sample of Merck's ouabain had an activity 75% that of the U. S. P. product, and the [α]_D was correspondingly less (-22.6°).

Antiarrin is the active principle of the latex of *Antiaris toxicaria* of Java, and is used as an arrow poison. It is isolated by the addition of 1.5 vols. of 95% alcohol to the latex, followed by filtration, evaporation and extraction of the residue with water. This solution is precipitated with lead acetate, the filtrate freed from lead and

evaporated. Kiliani (*Ber.*, 1910, 43, 3574) has shown that two glucosides are present in the latex; these he terms α - and β -antiarin.

α -*Antiarin* crystallises in shining colourless plates, m. p. 220 to 225°, and probably has the composition $C_{27}H_{42}O_{10}, 4H_2O$. Emulsin is without action on it, acids hydrolyse it with decomposition, the most suitable agent being a mixture of 8 parts 50% alcohol and 2 parts strong hydrochloric acid at 70°.



Antiarose is a methyl-pentose. *Antiargenin* crystallises in lustrous needles, m. p. 180°; the constitution is not yet known.

β -*Antiarin* forms slender needles or bunches of columnar needles, m. p. 206 to 207°. It is neutral and has the probable composition $C_{27}H_{38}O_{10}$ or $C_{28}H_{38}O_{10}, 3H_2O$. It is a glucoside, emulsin is without action on it, and the products of acid hydrolysis have not yet been isolated. There is apparently no difference in the toxic character of the two glucosides. Antiarin acts as a muscle and heart poison and closely resembles digitalis.

Windaus and Welsch (*Arch. Pharm.*, 1908, 246, 504) have obtained a crystalline substance, $C_{39}H_{56}O_2$, from the resin of *A. toxicaria*. This has m. p. 176° and is hydrolysed to α -amyrin and cinnamic acid.

Saponins¹

The saponins are a group of glucosides, very widely distributed throughout the vegetable kingdom, which possess the common property of forming a clear frothy solution in water, which forms emulsions with oils and resinous substances. They have a biting taste and in powder form cause sneezing. They are toxic, particularly to cold-blooded animals such as frogs and fish, and hæmolyse the red corpuscles of the blood. Live yeasts cannot be stained in their presence, but in the absence of salts of the alkali metals, are stimulated to an increased rate of sugar fermentation owing to an increase in the rate of intercellular diffusion. The effects of the different varieties on yeasts may be correlated with their hæmolytic activities. Gutbier, Huber and Haug (*Kolloid Z.*, 1921, 29, 19, 25) discuss the stability and behaviour of saponins as protective colloids. They are most stable in dilute solutions, and are affected relatively

¹ See article *Saponins* in Abderhalden's *Handbuch der biochemischen Arbeitsmethoden*, 1924 and addendum, p. 78.

little by the addition of reagents. They have little diuretic capacity, but, since they increase the amount of cholesterol in blood serum when injected intravenously, they have been used in cases of bubonic plague. The efficacy of sarsaparilla root in this respect has long been known.

The majority are homologous substances having the general formula $C_nH_{2n-8}O_{10}$. They vary considerably in the intensity of their toxic action, the more powerful being spoken of as *sapotoxins*. They are hydrolysed by dilute acids to dextrose, galactose, pentoses and substances called *sapogenins*, which are usually more active than their saponins and are often polyhydroxy lactones.

They are classified chemically into neutral and acid saponins, and are usually white or cream coloured, and insoluble in ether, benzene, chloroform, or cold ethyl alcohol. They are mostly protective colloids and are not dialysable, though digitonin, parillin and sarsaponin have been crystallised.

The more important plants which contain active saponins are:¹

Saponaria officinalis, 4 to 5% sapotoxin.

Quillaia saponaria, 9% sapotoxin.

Polygala senega, 2.5% sapotoxin.

Sarsaparilla (smilax), see p. 5.

Hemidesmus.

Agrostemma githago, 6.5% sapotoxin.

Digitalis purpurea (digitonin).

Claviceps purpurea (ergotinic acid).

Hedera helix (ivy).

The first two are the most active, senega saponin is only one-eighth as active, and digitonin still less so. With the exception of agrostemma, they are but little absorbed. They are powerful protoplasmic poisons and are strongly irritant. They are used as expectorants, and when administered by the mouth are excreted unchanged. Agrostemma (corncockle) saponin, being absorbed by both the subcutaneous tissue and the intestines, is a dangerous poison. The horse chestnut is also an important source of saponins. Winterstein and Moxim (*Helv. Chim. Acta.*, 1919, **2**, 195) compares them with those from *Sapindus saponaria*. They are isolated by extraction of the sample, de-fatted with ether in warm 95% alcohol

¹ See also Luft, *Monatsh.*, 1926, **47**, 259.

and calcium carbonate, this extract then being evaporated *in vacuo*, diluted, precipitated with lead hydroxide, and any lead removed from solution after filtration. The saponin, which is purified by dialysis and finally by acetylation with acetic anhydride in the presence of zinc chloride, is hydrolysed more readily than *Sapindus* saponins, and is a mixture of saponins, dextrose, *d*-fructose, arabinose and rhamnose being produced on hydrolysis. Vadas (*Chem. Ztg.*, 1927, 51, 895) points out that the removal of the saponin is of importance when the horse chestnuts are to be used in the starch or fermentation industries. The sample he examined contained 11.9% of saponin.

Saponins are detected in plants by the violet coloration given with concentrated sulphuric acid and the bluish-green colour formed with a mixture of equal parts of alcohol and sulphuric acid with a drop of ferric chloride. Some saponins, in the colloidal but not in the crystalline state give a blue adsorption compound with iodine. Mitchell (*Analyst*, 1926, 46, 181) has shown that commercial saponin (B. D. H.) may replace brucine or strychnine in the test for nitrates, though it is not known if this property is general or only specific for one saponin. The test, which may be used *inter alia* for saponin, is the production of a red colour on the addition of a drop of concentrated sulphuric acid to a mixture of saponin and the nitrate.

Preparation and Estimation of Saponins

Originally, hot alcohol was used for extraction, but this gives very impure products. Rochleder, also Christophson, precipitate aqueous extracts with hot saturated barium hydroxide, decompose this precipitate with carbon dioxide and precipitate the glucoside with ether-alcohol. Strütz purifies saponins by conversion into the acetyl derivative, hydrolyses this with barium hydroxide and decomposes with carbon dioxide.

Kobert and Pachorukow add excess of neutral lead acetate to the aqueous extract, whereby acid saponins such as quillaic acid are precipitated. Basic lead acetate is then added to the filtrate to precipitate the neutral saponins. Both precipitates are washed with alcohol, decomposed with sulphuric acid, and the lead removed. The saponin solutions are evaporated, the residue dissolved in alcohol, colouring matters are precipitated by chloroform, and the saponins precipitated by ether. Purification with baryta or by the acetyl

method yields physiologically inactive substances, for which Kobert suggests the name saponin, reserving sapotoxin for the poisonous substances. The ash content may be reduced by electro-dialysis.

The baryta compound is used for the *estimation of saponin*. The substance is extracted three times with boiling water, the extract concentrated, precipitated with alcohol and filtered, and the precipitate exhausted with boiling alcohol and added to the filtrate. After the removal of the alcohol the residue is dissolved in water, excess of saturated barium hydroxide solution added, and the precipitate collected, washed, and dried at 110°. It is weighed, converted by ignition into barium carbonate or into sulphate, and again weighed. This weight is calculated as BaO, and, subtracted from the first weight, gives that of saponin. When the plant material contains much starch it is advisable to extract with boiling alcohol instead of water. The extract is evaporated and freed from fat with ether.

As a check on the purity of the saponin-baryta precipitate it is dissolved in water containing a little hydrochloric acid, the barium removed quantitatively as sulphate, and the filtrate boiled. The sapogenin is collected, washed, dissolved in boiling alcohol ($D = 0.855$), the solution filtered, evaporated, and dried at 110°.

To determine saponins which are not precipitated quantitatively by baryta, May advises the addition of magnesium carbonate and extraction with methyl alcohol. The extract is evaporated, the residue boiled with ethyl alcohol and the saponin precipitated by ether.

The determination of the proportion of the products of the hydrolysis of the saponins is the most valuable method. Krushal dissolves in water, adds 2 c.c. hydrochloric acid per 100 c.c., and heats in sealed tubes for 3 hours at 100 to 140°. The sapogenin formed is collected on a tared filter, washed and dried at 100°, carbohydrates being determined in the united filtrate and washings, and Nicoresti and Tallantyre (*Pharm. J.*, 1923, **III**, 103) use a similar method. One part of sapogenin is equivalent to 3.22 parts of saponin. It is suggested that an alcoholic tincture should contain a minimum of 1.8% of saponin, as determined by this method. A sample of *Q. saponaria* was found to contain water-soluble constituents, 32.4; sapogenin, 3.35 (*i. e.*, 10.78% of saponin); alcohol-soluble constituents, 22.25; and ash 9.05%.

Cholesterol is an antidote for saponins, yielding poisonous double compounds with varying degrees of dissociation. That of digitonin is crystalline and characteristic (see p. 39), and Kofler (*Chem. Ztg.*, 1924, 48, 702) has suggested a method of determination based on the increase of cholesterol in blood serum after intravenous injection of the saponin.

The bark of *Quillaia saponaria* (soap bark) contains two toxic glucosides—quillaic acid and sapotoxin.¹ Commercial saponin is a mixture of these two with inert matter which consists mainly of a non-poisonous modification of quillaic acid, and is termed by Kobert saponin.

Quillaic Acid, $C_{19}H_{36}O_{10}$, is prepared by precipitating the aqueous extract of the bark with neutral lead acetate. The lead is removed, the clear solution evaporated almost to dryness, the residue extracted with boiling absolute alcohol, and chloroform added to precipitate the colouring matter. The glucoside separates in white amorphous flakes, soluble in water or alcohol, but insoluble in ether. It becomes dark red on treatment with strong sulphuric acid. On hydrolysis it yields the sapogenin $C_{29}H_{46}O_5$, which is probably related to the stigmasterol group of the bile acids.

When quillaic acid is purified by the baryta or acetyl processes the physiological activity is entirely lost, but the chemical properties are unchanged.

Quillaia Sapotoxin, $C_{17}H_{26}O_{10}$, is contained in the filtrate from the above-mentioned lead acetate precipitate. It is a colourless amorphous powder possessing all the poisonous properties of the sapotoxins and resembles quillaic acid in solubility. Concentrated sulphuric acid dissolves it, with a yellow coloration which slowly becomes yellowish-red.

Saporubrin, $(C_{18}H_{28}O_{10})_4$, the sapotoxin of *Radix saponariae rubrae* from *S. officinalis*, is an amorphous neutral powder which causes sneezing when inhaled. It tastes sweet at first, but afterward sharp and acid; it is readily soluble in water and dilute alcohol, sparingly so in absolute alcohol. It dissolves in concentrated sulphuric acid, with a reddish-brown colour, becoming reddish-violet on

¹ Other constituents of the bark are starch and gum. The ash is about 8 to 14%. Quillaia bark is official in the British Pharmacopœia, and is the dry inner portion of the bark. It is used in the form of an infusion or tincture, and the ash should not exceed 15%. The botanical characteristics of the chief species required by the various Pharmacopœias are described by Nicoresti and Tallantyre (*vide supra*).

exposure to the air and emerald green on the addition of a few drops of potassium dichromate. It has $[\alpha]_D - 54^\circ$.

On hydrolysis a series of sapogenins is obtained, those first formed giving up another molecule of sugar on continued action of the dilute acid. The amount of sugar obtained varies from 60.27 to 63.9%, and that of sapogenin from 30.2 to 34.2%, the final product having the formula $C_{14}H_{22}O_2$.

Levant Sapotoxin, $(C_{17}H_{26}O_{10}, H_2O)_2$, is obtained from Levantine soap wort, *Radix saponariæ albæ*, derived from *Gypsophila arrostii* or *G. paniculata* by the general method of extraction described on page 54. The root does not contain a saponin. The sapotoxin is very similar to saporubrin in physiological and chemical behaviour; it dissolves in fuming nitric acid, with a yellow coloration, changing to green on the addition of potassium dichromate. On hydrolysis, four molecules of sugar (*i. e.*, 56%), probably a mixture of glucose and galactose, and a *sapogenin*, $C_{10}H_{16}O_2$ (23%), are formed.

Agrostemma-sapotoxin, $(C_{17}H_{26}O_{10})_2$, from the latex of corncockle, *Lychnis* or *Agrostemma githago*, is a yellowish-white amorphous powder, not precipitated by neutral lead acetate, and having the characteristic properties of the group. It yields four molecules of sugar and a sapogenin, $C_{10}H_{16}O_2$, on hydrolysis, and is very poisonous (see p. 53).

Senega root from *Polygala Senega* contains two glucosides—*polygalic acid* which is precipitated by neutral lead acetate, and a sapotoxin, *senegin*, $C_{17}H_{26}O_{10}$. These are very similar to the quillaia bark glucosides, but not identical with them. Concentrated nitric acid dissolves polygalic acid, with a ruby-red coloration, and senegin, with a golden-yellow coloration. The drug contains a small quantity of methyl salicylate which increases on storage, probably owing to a gradual decomposition of a glucoside. The drug yields about 4% of ash. Brandl (*Arch. exp. path. Pharm.*, 1908, **59**, 245) has shown that the lead acetate precipitate contains a second saponin, *agrostemmic acid*, of higher molecular weight than the sapotoxin, but having the same toxic effects. It yields *sapogenin*, $C_{35}H_{54}O_{10}$, dextrose and galactose, with possibly some arabinose, on hydrolysis.

The glucosides of sarsaparilla root include:—

Parillin, $C_{26}H_{44}O_{10}$, a colourless crystalline powder, sparingly soluble in cold water, but soluble in 20 parts of boiling water. It readily dissolves in alcohol of about sp. gr. 0.830. It is obtained

from the root by extraction with alcohol and precipitation of the extract with water. The dry powder is colourless, and in aqueous solution has a neutral reaction, froths strongly, and has a sharp bitter taste. It has m. p. 177° , $[\alpha]_D - 42^{\circ}$, and forms a *penta-benzoyl* derivative, m. p. 76° . On hydrolysis, which is best carried out under pressure, a mixture of a phytosterolin $C_{33}H_{56}O_6$ (which is hydrolysed to dextrose and to sitosterol) and *parigenin*, $C_{28}H_{46}O_4$, is formed. The latter is a colourless crystalline insoluble substance, which on oxidation with nitric acid yields picric, benzoic and oxalic acids. More recent work (see below) has indicated that it is a mixture.

Smilasaponin, $(C_{20}H_{32}O_{10})_5$, previously called *smilacin* or *sarsaparil saponin*, is obtained by extraction with boiling water and purified by precipitation with baryta. It is a yellowish horny mass which resembles starch flour when powdered. It is laevorotatory. Acids hydrolyse it to a *sapogenin*, $C_{28}H_{46}O_4$. Like parillin, it forms a *pentabenzoyl* derivative.

Sarsasaponin, $(C_{44}H_{76}O_{20})$, is obtained from the aqueous alcoholic mother liquors after precipitation of parillin. It crystallises in long broad needles of silky lustre, m. p. 248° , $[\alpha]_D - 48.5$. It is readily soluble in water and in absolute alcohol and forms a *tetra-benzoate*. Acids hydrolyse it to dextrose and *sarsasapogenin*, $C_{26}H_{42}O_3$. In its chemical behaviour it is similar to parillin. Physiologically, sarsasaponin is the most active of the sarsaparilla glucosides, the lethal doses of parillin and smilasaponin being three or four times as large. In addition to the glucosides, sarsaparilla contains varying quantities of starch and about 7% of ash (see also p. 56). Varieties are Jamaica, Lima, Honduras, Guayaquil and Mexican. The Honduras variety is more starchy than the Jamaican.

Sarsaparilla root has been fully investigated by Power and Salway (*J. Chem. Soc.*, 1916, 105, 201), who worked with the grey Jamaica root of the British Pharmacopœia (*Smilax ornata*). The following compounds were obtained from the alcoholic extract:

- (1) *Sarsasaponin*, (*vide supra*).
- (2) *Sitosterol-d-glucoside* (a phytosterolin)— $C_{33}H_{56}O_6$ (m. p. $280-285^{\circ}$).
- (3) *Sitosterol*, $C_{27}H_{46}O$ (m. p. $135-136^{\circ}$, $[\alpha]_D - 27.3^{\circ}$).
- (4) *Stigmasterol*, $C_{30}H_{50}O$, which is identified by its tetrabromoacetyl derivative, $C_{30}H_{49}OBr_4.COCH_3$ (m. p. 208°).

(5) *Sarsapic Acid*—a crystalline dicarboxylic acid (m. p. 305°), $C_4H_2O_2(CO_2H)_2$.

(6) *Dextrose*.

(7) *Fatty Acids*—viz., palmitic, stearic, behenic, oleic and linolic acids.

(8) *Cetyl-d-glucoside*. (?)

(9) Potassium nitrate.

(10) 1.25% of resins.

The root contains a small quantity of an enzyme of the emulsin type.

Inasmuch as only one definite saponin glucoside is present, it was thought probable that the parillin of earlier investigators consisted of a mixture of sarsasaponin and a phytosterolin. Commercial smilacin represents a relatively small portion of sarsasaponin with indefinite amorphous products. Honduras sarsaparilla root was found by Kaufmann and Fuchs (*Ber.*, 1923, **56**, [B], 2527) to contain a sarsasaponin obtainable by extraction in succession with chloroform, petroleum spirit, benzene, ether, anhydrous and aqueous (50%) alcohol, and hydrolysed to sarsasapogenin, $C_{26}H_{42}O_3$, H_2O , m. p. 183° .

Polysciasaponins.—These occur in the leaves of *Polyscias nodosa* and have been separated by Van der Haar (*Pharm. Zeit.*, 1908, **53**, 900) by fractional crystallisation into α - and δ -types ($C_{22}H_{36}O_{10}$ and $C_{26}H_{42}O_{10}$, respectively). They are white amorphous powders, hydrolysed to arabinose, dextrose and a sapogenin $C_{26}H_{44}O_4$ (a saturated lactone containing no hydroxyl or allied groups).

Hederin.—Van der Haar (*Ber.*, 1921, **54**, [B], 3142) investigated the hederins or saponins of *Hedera helix* (ivy), which occur as an α - and β -hederin (crystalline, insoluble in water), γ -hederin (amorphous, insoluble), and δ -hederin (soluble in water). Jacobs (*J. Biol. Chem.*, 1925, **63**, 621 *et seq.*) found the sapogenin from *Sapindus mukorossi utilis* to be the same as hederagenin, the formula and properties of which he found slightly different to Van der Haar.

α -Hederin $C_{42}H_{66}O_{11}$, m. p. 256° , $[\alpha]_D^{20}$ 9.68 is hydrolysed to α -hederagenin $C_{31}H_{50}O_4$, m. p. 325° (a dihydroxy lactone), arabinose and rhamnose. The hederins form sodium and potassium salts and are reduced to a mixture of sesquiterpenes ($C_{15}H_{24}$) which give a violet colour with sulphuric acid, and a non-volatile solid.

Mowrin $C_{42}H_{68}O_{25}$, in the alcoholic extract from mowrah seeds (*Bassia longifolia*) is hydrolysed to lævulose, arabinose and mowric acid ($C_{19}H_{28}O_5 + C_{19}H_{30}O_6$).

Fatsin $C_{31}H_{53}O_{20}$ has recently been isolated from *Fatsia japonica* by Ohta (see *Brit. Chem. Abs.*, 1928, A, 48). It is hydrolysed to a mixture of α - and β -sapogenins, a sugar, and an organic acid.

The following less important saponins are given in the Table on p. 6:—**Caulosaponin**, $C_{54}H_{88}O_{17}$, and **Caulophyllsaponin**, $C_{66}H_{104}O_7$, were found (Power and Salway) in *Caulophyllum thalictroides*. **Jegosaponin**, $C_{75}H_{80}O_{25}$ (*Styrax japonica*).

The alcohol extract of alfalfa hay contains an amorphous saponin $C_{26}H_{37}O_{16}N$ (decomposes at $280-300^\circ$) which is abnormal in that it contains nitrogen and does not hæmolyse blood. It is toxic to fish, however, and is a powerful frothing agent. The root of *Yucca radiosa* contains the saponin $C_{37}H_{58}O_{20}$, and that of *Y. angustifolia* $C_{36}H_{56}O_{20}$. Glasa and Krauter (*Ber.*, 1924, **57**, [B], 1064) isolated 2 saponins from *Polygala amara*: (a) the neutral saponin $C_{34}H_{52}O_{20}$, $[\alpha]_D$ 30, hydrolysed to dextrose (3 mols.) and the sapogenin $C_{14}H_{22}O_2$; (b) the amorphous acid saponin $C_{22}H_{38}O_{10}$, yielding dextrose and the sapogenin $C_8H_{14}O_3$. Varieties of *Phoenix* also contain saponins combined with calcium or magnesium. Convolvulin and jalapin (see p. 63) also are analogous to saponins in that (1) they have hæmolytic effects in neutral, though not in alkaline solutions, (2) they are toxic to fish and (3) they are incompletely absorbed in the alimentary tract. The relation of digitonin to the saponins has already been indicated (p. 38).

Detection of Saponin in Beverages and Foods

Saponins are sometimes added to beers, sparkling wines, lemonade, etc. to induce frothing. Kobert's original procedure was to extract with *isobutyl* alcohol.

Vamvakas' test (*Z. Unters. Nahr. Genussm.*, 1907, **13**, 271), which depends on the formation of a dirty grey-coloured precipitate when a saponin solution is treated with a drop of Nessler's reagent, has been condemned as not specific by numerous workers.

Frehe (*Z. Unters. Nahr. Genussm.*, 1899, **2**, 938) evaporates the beverage to dryness, extracts the residue with ethylacetate, and tests the extract for a colour reaction with sulphuric acid, but Rühle (*loc.*

cit.) and Behre (*loc. cit.*) have shown that, since saponin is almost insoluble in ethyl acetate, the method has little value.

Brunner (*Z. Unters Nahr. Genussm.*, 1902, 5, 1197) saturates with magnesium carbonate and extracts with phenol. This extract is then poured into water, and the phenol removed by means of ether. This method has been generally approved (Rühle, *ibid*, 1908, 16, 165).

The latter (*ibid.*, 1914, 27, 192) has therefore evolved a method unaffected by artificial dyes used in effervescent beverages, which has been modified by Campos (*Annali. Chim. Appl.*, 1914, 19, 289). The sample (500 c.c.) is evaporated to 10 c.c. on the water-bath and shaken with 10 c.c. of melted phenol and 20 grm. of ammonium sulphate. The saponin is removed from the phenol layer by shaking it with 100 c.c. of ether and 50 c.c. of water, and the aqueous layer evaporated to 25 c.c. and shaken with successive 100 c.c. portions of a fresh solution of barium hydroxide till colourless, the clear liquid being decanted each time. The united precipitates are decomposed in a slow current of carbon dioxide, and the liquid, after filtration, evaporated and extracted 4 times with boiling alcohol. This extract is evaporated and tested with sulphuric acid. If the saponin is impure, colours ranging from green and yellow to red and violet may be obtained.

Stoecklin (*Ann. Falsif.*, 1917, 10, 561) showed that flour may be poisoned by the presence of corn cockle (*Agrostemma githago*), which sometimes occurs in it. The dried flour is extracted at 45° for 45 minutes with 15 c.c. of a sterile 0.95% salt solution, the extract filtered, and 0.5 c.c. of blood emulsin added. This is prepared from fresh ox blood, shaken with broken glass till defibrinated, and 25 c.c. centrifuged with 225 c.c. of sterile 0.95% salt solution for 45 minutes. The liquid is decanted, and a further 250 c.c. of salt solution added. If saponin is present, the liquid becomes clear in a time proportional to the amount present. Comparison may be made with known amounts of pure quillaia saponin, and the test confirmed by microscopical examination of the flour.

Kofler (*Chem. Ztg.*, 1924, 48, 165, 702) pointed out that, on account of the wide variations in purity of different saponins, no parallel exists between the hæmolytic powers, the frothing powers, and the fish index, (the concentration required to kill a young roach 0.5 grm. in weight in 1 hour). This is important in practice, where the use

of an increased quantity of saponin of low frothing power may lead to an increase in toxicity altogether out of proportion with the amount added. This is illustrated by the table below. He therefore suggests the use of the ratio, hæmolytic index \div froth number for assessing the value of saponins for use in foodstuffs. The frothing power is measured by the addition of sufficient water or physiological salt solution to 1, 2, 3 . . . 10 c.c. portions of a 0.1% solution of saponin in the same solvent contained in test-tubes, 16 mm. in diameter, to produce 10 c.c. of liquid. These are corked, and well shaken for 15 seconds, when the frothing power is given by the dilution of saponin which gives a froth 1 cm. high, permanent for 15 minutes. The hæmolytic index is determined on 5 c.c. of fresh defibrinated rats' blood diluted with 50 times its volume of physiological salt solution and mixed with 1, 2, 3, 4 or 5 c.c. of a 0.1% solution of saponin in the same solvent. The volumes are then made up to 10 c.c., the solutions mixed by one rotation, and the dilution of the first tube in the series showing hæmolysis (a red coloration with intact blood corpuscles on the bottom of the vessel) gives the required index, (*Z. Unters. Nahr. Genussm.*, 1922, **43**, 278). It is suggested that the ratio should not exceed 0.5–1.0 for foodstuffs. Mand (*Chem. Ztg.*, 1926, **50**, 850) has criticised these conclusions. He points out that the ratio varies widely for the different saponins (0.023–10.0), and suggests that, since the relative hæmolytic and toxic effects may also vary considerably for different saponins, a more useful figure is given by the expression, hæmolytic index \times toxicity \div froth number. These values are compared in the following table for a number of saponins.

Variety of saponin	Hæmolytic index	Froth no.	Hæmolytic index \div froth no.
Guaiacum saponin (Merck).....	660	28,500	0.023
Sapotoxin (Merck).....	30,000	20,000	1.5
Sapindus saponin (Hoffmann-La-Roche).....	28,600	16,700	1.7
Aphrogen.....	2,000	800	2.5
Horse chestnut saponin (Merck)....	10,000	3,300	3.0
Sap. pur. albiss. (Merck).....	25,000	2,500	10.0
Quillaia saponin.....	32,000	10,000	3.2
Spumagen (Mand).....	2,000	3,700	0.5

In conjunction with Lázár (*Arch. Pharm.*, 1927, **265**, 610), Kofler¹ has recently shown that saponins may be divided into three classes

¹ See addendum, p. 78.

depending on the effect of the pH value on their hæmolytic powers. In determinations of the latter it is therefore important that the pH value should be standardised. For example, very different results were obtained with washed in place of unwashed blood corpuscles.

Glucosides of Jalap and Scammony

Jalap and Scammony and several allied plants of the *Convolvulaceæ* yield glucosides of a complicated nature which contain in particular fatty residues. They are mostly drastic purgatives. The active principles of Jalap and Scammony are often miscalled resins.

Convolvulin, $C_{54}H_{96}O_{27}$, is the active principle and constitutes 90% of true Jalap, the root or tuber of *Ipomœa purga*. It is a colourless, amorphous powder, m. p. 150° , very sparingly soluble in water or ether, but readily soluble in alcohol and acetic acid. Concentrated sulphuric acid dissolves it with a red coloration. It is hydrolysed by mineral acids to dextrose, and *convolvulinic acid*, $C_{15}H_{30}O_3$, which is subsequently converted into rhamnose and rhodose (a methyl pentose, $C_6H_{12}O_5$). This acid yields on oxidation methylethylacetic acid and *ipomic acid*, $C_{10}H_{18}O_4$, which is an isomeride of sebacic acid.

Jalapin and **Scammonin**, $C_{44}H_{56}O_{16}$, are identical. This glucoside is the active principle of the root stalk of *Ipomœa orizabensis*¹ and of Scammony, the dried sap of *Convolvulus scammonia*. It is an amorphous resinous powder, translucent in thin plates, m. p. 131° , $[\alpha]_D - 23^{\circ}$. It is slightly soluble in water, readily in alcohol, ether, chloroform or benzene. Concentrated sulphuric acid dissolves jalapin, with a purple or maroon colour, changing to brown and finally becoming black. Acids hydrolyse it to dextrose (3 mols.) and *jalapinolic acid*, $C_{15}H_{30}OH.CO_2H$, m. p. $67-68^{\circ}$; this acid is slightly *d*-rotatory and yields methylethylacetic acid, sebacic acid and an isomeride of this on oxidation. The analogy between convolvulin and jalapin and the saponins has already been mentioned (see p. 60 and Heinrich, *Biochem. Z.*, 1918, 88, 13).

Jalap

Commercial jalap consists of the dried tubercles of *Ipomœa purga*. It occurs in pieces about the size of an egg which, however, vary

¹ The work of Power and Rogerson throws doubt on the existence of jalapin as a chemical individual (see p. 68, note and *J. Amer. Chem. Soc.*, 1910, 32, 112), and indicates that the physiological constituents of jalap and their hydrolysis products are complex and indefinite.

in length (2.5 to 7.5 cm.) and shape, the larger roots being cut. Externally they are dark brown, furrowed and wrinkled and are marked with transverse scars. They break with difficulty. The internal surface is yellowish-grey or dull brown, and a transverse section shows concentrically arranged irregular dark lines. The drug at first tastes sweet, then acrid and disagreeable.

The principle constituent is the glucoside resin, but the root also contains sugar, starch, proteins and calcium oxalate present in the form of crystals in clusters of rosettes. In other convolvulaceous roots the calcium oxalate crystals are generally acicular (Greenish), a characteristic which may be used to distinguish jalap. The drug yields about 6% of ash. The amount of resin varies from 5 to 18%, or generally from 8 to 12%. According to the British Pharmacopœia it should yield not less than 9 nor more than 11%, and should contain not more than 6.5% of ash. Jalap, United States Pharmacopœia, should contain not less than 7% total and not more than 1.5% ether-soluble resin. The dry tuberous root of *Exogonium Jalap* is given as the source, and the assay is made on 10 grm. of the powdered root heated under a reflux condenser for 3 hours with 50 c.c. of alcohol. The mixture is allowed to percolate, and alcohol added till exactly 100 c.c. of tincture are obtained. To 20 c.c. of this tincture are added 10 c.c. of chloroform and 20 c.c. of a saturated solution of potassium citrate (20 grm. in 12 c.c. of water), and the whole shaken for 2 minutes. After not less than 10 hours the alcohol-chloroform layer is removed, and filtered into a tared flask, a mixture of 10 c.c. of alcohol and 5 c.c. of chloroform being used for the purpose of washing and rinsing. The filtrate is evaporated and the residue dried at 100° and weighed. Greenish states that the quality of the drug has deteriorated recently, 12 to 18% being formerly the usual amount of resin present.

Samples analysed by Evans, Sons, Lescher and Webb in 1907 to 1910 contained from 7.4 to 11% of resin. The tubers usually contain from 12 to 16% of moisture. They extracted the resin from the drug in 60-mesh powder by boiling with 95% alcohol.

Moore (*J. Soc. Chem. Ind.*, 1904, 23, 412) gives the average resin content of 98 samples of jalap (bought in America) as 12.6%. Subsequently (*ibid.*, 1906, 25, 627) 276 samples were examined only 15 of which were equal to or above 11%. The average was

5.95% of resin, showing that low grade jalap is of frequent occurrence.

The glucoside resin is a mixture of 2 glucosides, one, about 10% of the whole, being soluble in ether, and the other, *convolvulin*,¹ about 90% of the whole, being insoluble in ether, but soluble in alcohol. The soluble resin is identical with that from scammony root and is termed *scammonin* or *jalapin*.

Tampico Jalap, the root of *I. simulans*, is distinguished by its irregular shape and convoluted surface, which does not show the transverse scars characteristic of true jalap. It contains about 10% of ether-soluble resin (*scammonin*).

Orizaba Jalap (Mexican scammony, *orizabin*), the root of *I. orizabensis*, occurs mostly as transverse slices 5 to 10 cm. long and 1.5 to 2 cm. thick, evidently portions of a large root divided. It contains 17 to 18% of resin (*scammonin*) soluble in ether, and serves in Germany as the main source for making the resin which is termed there *jalapin*.

Jalap Resin

Jalap resin is prepared by digesting 100 parts of jalap with twice its weight of 90% alcohol for 24 hours at a moderate temperature, transferring the mixture to a filter, allowing the liquid to pass and exhausting by percolation with more alcohol. The alcohol is removed by distillation, and the residue poured into 8 times its weight of distilled water. The separated resin is dried after washing with cold water, (see Rosenthaler, *Arch. Pharm.*, 1925, 263, 561).

According to the British Pharmacopœia it (*Jalapæ Resina*) occurs in dark brown opaque fragments, translucent at the edges, brittle, readily reduced to a pale brown powder, acrid in taste and sweetish in odour. It dissolves readily in 90% alcohol, but is insoluble in oil of turpentine, and 1 grm. triturated with 20 c.c. of water should yield a colourless filtrate. To prove the absence of scammony resin and resin of Tampico jalap, the powder should yield practically nothing to warm water and not more than 15% to ether, and a solution of 0.1 grm. in 10 c.c. of sodium hydroxide solution, boiled, cooled and acidified with hydrochloric acid should give no more than an opalescence at first. In the absence of gualiacum resin a

¹ The British Pharmaceutical Codex restricts the name *jalapin* to the glucoside termed *convolvulin* above, but the latter term has found more general use in scientific literature and will be adhered to here.

solution in alcohol is not coloured bluish green by ferric chloride solution. It is used in the form of a mixture with 2 parts of potassium hydrogen tartrate and 1 part of ginger. The tincture is prepared from 200 grm. of jalap, ground to pass a No. 40 sieve, and mixed with 70% alcohol. The solution is diluted till 1.5% in strength, and 10 c.c. concentrated by evaporation and poured into 8 times its volume of water, should then cause a separation of 0.145–0.155 grm. of (dry) resin. Similar tests are required by the U. S. Pharmacopœia. In the absence of guaiacum resin, a solution in alcohol is not coloured bluish-green by ferric chloride solution.

W. B. Cowie (*Pharm. J.*, 1908, **81**, 363) recommends the following tests: (1) Moisture. (2) Ash. (3) Solubility in ether.—1 grm. of the powdered resin is rubbed in a mortar with 10 c.c. of ether, the ether filtered through cotton wool into a tared flask, and the operation repeated thrice. The ether is distilled and the residue weighed after drying at 110°. (4) Acid value.—1 grm. of resin dissolved in 30 c.c. of alcohol is used. (5) Saponification value.—The resin is boiled for 1 hour with 0.5/*N* alcoholic potassium hydroxide and titrated with 0.5/*N* hydrochloric acid. (6) Absence of colophony.—0.25 grm. of resin in 5 c.c. acetic anhydride should give no purple colour with 2 drops of strong sulphuric acid. (7) Absence of guaiacum.—No greenish-blue colour with ferric chloride. (8) Resin yields no water-soluble substance and is free from bitterness. Dale (*Pharm. J.*, 1927, **119**, 516) determines the resin by the complete extraction of the powdered jalap with 100 c.c. of hot alcohol. The filtered extract is concentrated to 50 c.c., re-filtered, and evaporated. The residue is digested for 2 minutes at 65° with 15 c.c. of water, cooled, the liquid decanted on to an 11 cm. filter-paper, and the residue washed by decantation with 15 c.c. portions of water at 65°. The combined residues are then dissolved in 15 c.c. of alcohol, filtered into a tared beaker, the filter being washed successively with two 10 c.c. and one 5 c.c. portions of hot alcohol, filtrate and washings evaporated, and the residue weighed.

The ether-soluble resin—convolvulin—has acid value 2.8, saponification value 408, $[\alpha]_D - 39.5^\circ$. Commercial "white jalap resin" gave: moisture 3%, ash 0.02%, ether-soluble 0.3%, acid value 2.8, saponification value 417. Two samples of brown jalap resin gave: moisture 5.0 and 5.6%, ash 0.3%, ether-soluble 10%, acid value 11.2 and 14, saponification value 333 and 338.

Scammony Root

Scammony root is derived from *Convolvulus scammonia*, a climbing plant indigenous to the Eastern Mediterranean. The roots are often very large; small ones measure 6 to 12 in. long and 1 in. in diameter; large roots are 3 or 4 in. thick and 2 to 3 feet long. The transverse section shows a number of nearly circular bundles. The odour is characteristic, like that of jalap, and the taste acid.

On an average, the root contains 8% of glucoside resin (scammonin), starch, sugar and about 10% ash. The sugars comprise dextrose, sucrose, and small quantities of methyl pentose.

Scammony Resin

The resin is prepared by extraction of the root with 90% alcohol, removal of most of the alcohol by distillation, and precipitation of the resin by pouring the residual liquid into eight times its volume of water. The resin is collected and washed with boiling water, and dried at a low heat.

It occurs in pale brownish, translucent brittle pieces with a sweet odour and resinous fracture. It does not form an emulsion alone with water (a distinction from the scammony gum resin), is entirely soluble in ether, and the alcoholic solution gives no blue coloration with ferric chloride or hydrogen peroxide, showing the absence of guaiacum resin.

Scammonia resin, *S. radix* (from *Convolvulus scammonia*) and *Ipomæa* (Mexican Scammony) are now official either in the British or U. S. Pharmacopœias. In the former case not less than 75% should be soluble in ether, and the same tests are applied as in the case of jalap resin (*vide supra*). *S. radix* is grey to brown or yellow and shows dark resin cells on a pale ground of tissue. Starch grains are abundant in the parenchymatous tissue. The U. S. Pharmacopœia tests are the same as for jalap. The acid-insoluble ash should not exceed 3%.

Cowie (p. 66) examined scammony resin on similar lines to jalap. Two specimens of "white resin" were entirely soluble in ether and gave moisture 2.52 and 5.3%, ash 0.02%, acid value 2.8, saponification value 241, $[\alpha]_D - 25^\circ$, m. p. 120° . Pure brown scammonin had 4.5 and 5.1% moisture, 0.15% ash, acid value 25.2 and 28, saponification value 263; m.p. 100° . The limit for acid value is 8.4

for white resin, and 34 for brown. 95% of the brown resin should dissolve in ether.

Mexican scammony resin¹ from *Resina drastica* has been examined by Cowie and Brander (*Pharm. J.*, 1908, 81, 366). About 70% was soluble in ether, the acid value was 8.4, saponification value 295 to 327. 4.8% was soluble in carbon disulphide. It is used as a substitute for the incision product (Scoville, *J. Ind. Eng. Chem.*, 1919, ii, 335).

¹ A more complete investigation of "Mexican Scammony Root" (*Ipomaea orizabensis*) was published by Power and Rogerson (*J. Chem. Soc.*, 1912, 101, 1, 398) of which the following is a summary.

The root was found to contain 14.55% of resin, of which 71% was soluble in ether. After treatment with animal charcoal the resin was obtained nearly colourless; it then melted at 125–130° and had $[\alpha]_D = -23.0^\circ$.

For a complete examination of the root 48.76 kgm. were extracted with hot alcohol; a portion of the concentrated extract was used for determining the presence of sucrose, and a small amount of it was isolated. Another portion of the extract was steam-distilled, when a very small amount of a pale yellow essential oil was obtained. From the portion of the extract which was soluble in water the following compounds were isolated:

(i) *Scopoletin*, $C_{10}H_8O_4$ (m. p. 203–204°), a small portion of which appeared to be present in the form of a glucoside.

(ii) 3:4-Dihydroxycinnamic acid, $C_9H_8O_4$ (m. p. 223–225°) from which the methyl ester (m. p. 158–160°) was prepared.

The aqueous liquid also contained a quantity of sugar which yielded d-phenylglucosazone (m. p. 205–206°).

The portion of the alcoholic extract which was soluble in water consisted of the above-mentioned resin. The resin was extracted successively with various solvents.

I. *Petroleum Spirit Extract of the Resin*.—From this extract the following substances were obtained:

- (i) *hentriacontane*, $C_{31}H_{64}$;
- (ii) *A. phytosterol*, $C_{27}H_{48}O$;
- (iii) *Cetyl alcohol*, $C_{16}H_{34}O$;
- (iv) A mixture of fatty acids, consisting of palmitic, stearic, oleic and linolenic acids.

II. *Ethereal Extract of the Resin*.—This extract had $[\alpha]_D = -20.5^\circ$. After hydrolysis with barium hydroxide it gave:

- (i) *Ipuranol*, $C_{13}H_{26}O_2(OH)_2$; (ii) *d*- α -methylbutyric acid; (iii) *tiglic acid*; and a product which on acid hydrolysis gave (iv) *jalapinic acid*, $C_{11}H_{18}(OH)CO_2H$ (m. p. 67–68°; $[\alpha]_D + 0.79^\circ$), together with a little methyl jalapinolate and (v) a mixture of sugars consisting of dextrose and a *methylpentose*. The latter yielded an osazone, m. p. 180–182°, and a *tetraacetyl derivative*, $C_{11}H_{14}O_8Ac_4$, which apparently is a new compound, m. p. 142–143°, $[\alpha]_D = +21.64$. The ethyl acetate extract of the product resulting from the alkaline hydrolysis of the ethereal extract gave on oxidation with nitric acid, a mixture of acids, consisting apparently of optically active valeric and hexoic acids, together with sebacic and *n*-nonanedicarboxylic acids.

III. *Chloroform Extract of the Resin*.—This was relatively small in amount and consisted of a dark resinous product.

IV. *Ethyl Acetate Extract of the Resin*.—This extract had $[\alpha]_D = 28.01$. After hydrolysis with barium hydroxide it yielded products from which the same products were obtained as from the ethereal extract of the resin, with the exception of ipuranol.

V. *Alcoholic Extract of the Resin*.—This was a black amorphous product of a glucosidic nature, but which yielded nothing definite on hydrolysis.

It thus appears that the resin of *Ipomaea orizabensis* is an exceedingly complex mixture, the constituents of which are, for the most part, amorphous and not entirely glucosidic. It follows, therefore, that the so-called "jalapin" which includes all the constituents which are soluble in ether, cannot be represented by any of the various formulae hitherto suggested. The portion of the resin which is soluble in ether is not identical with the ether-soluble portion of jalap resin, as has previously been affirmed.

As a result of later experiments on "virgin" scammony resin, obtained by incision from the living root of *Convolvulus scammonia*, they concluded that it differed considerably from that obtained from the root of *I. orizabensis*. Both are very complex and consist largely of the glucosides and methylpentosides of jalapinic acid, but the former gives rhamnose on hydrolysis (in place of the tetraacetyl derivative obtained from the latter), and contains no hentriacontane or cetyl alcohol. Differences in solubility towards various solvents also exist. The above points of difference may not, however, be constant to such an appreciable extent as to influence the therapeutic value.

Taylor (*Amer. J. Pharm.*, 1909, **81**, 105) has published complete analyses of scammony resin from *Convolvulus scammonia* and of "Mexican Scammony" from *Ipomæa orizabensis*. The microscopical examination and determination of the saponification value appear to afford means of distinguishing the two resins, (see Ballard, *Pharm. J.*, 1912, **34**, 285 and Bourdier, *J. Pharm. Chim.*, 1912, **5**, 251).

Scammony

Scammony is the gum resin obtained by incision from the living root. The emulsion is collected in shells and allowed to dry. It is usually imported in flattened cakes varying in colour from dark grey to brown or nearly black. Thin fragments are translucent and the fractured surface is glossy. It has a cheesy odour. It is easily reduced to an ash-grey powder and yields a milky emulsion when rubbed with water.

Pure scammony contains as much as 88 to 90% of ether-soluble resin, the residue consisting of gum. Commercial qualities, known as "virgin," usually vary from 75 to 85%, the limit being 70% of resin, though it is no longer official in the British and U. S. Pharmacopœias. It should not contain more than traces of starch and the ash must not exceed 3%.

Adulteration.—Scammony is frequently adulterated with starch, chalk, and earthy matter, or with other resins. Foreign starches may be detected microscopically. Excess of ash will show inorganic matter. To detect resins the ether cal extract is dissolved in a hot solution of potassium hydroxide (see p. 79). Scammony resin is not precipitated on acidification, whereas most foreign resins are. It is now largely replaced by Mexican jalap.

Inferior scammony is usually tough, has a dull fracture, and small splinters are opaque. A variety sold as skilleep usually consists of flour dough mixed with some of the gum resin. Evans, Sons, Lescher and Webb report on samples labelled "Aleppo" which contained 20 to 23.8% of ether-soluble matter, instead of about 50%. Nine samples of genuine Aleppo scammony had a range of 57.2 to 66.5% of resin.

Coumarin Glucosides

Coumarin is widely distributed among plants, probably as a glucoside. Glucosides containing hydroxy coumarin, however, are

definitely known. The most important are described below and in the Tables on pp. 6-9.

Skimmin, $C_{15}H_{16}O_8$, is a constituent of *Skimmia japonica* and the glucoside of skimmetin (4-hydroxy-coumarin), an isomer of umbelliferone.

Aesculin, $C_{15}H_{16}O_9$, is found in the horse chestnut (*Aesculus hippocastanum*) and is the glucoside of aesculetin (dihydroxy-coumarin). It is an isomer of *Daphnin*, a glucoside of daphnetin; its monomethyl ether is *Scopolin*, $C_{22}H_{28}O_{14}$, a glucoside from *Scopolica japonica*, and its dimethyl ether is *Limettin* which is found in the citrus.

Fraxin, $C_{16}H_{18}O_{10}$, found in the ash, in species of *Aesculus* and in *Dienvilla*, is the glucoside of fraxetin (a monomethyl ether of tri-hydroxy-coumarin).

Phytosterolins.—A number of glucosides of phytosterols have been isolated from plants, of which sitosterol, $C_{33}H_{56}O_6$, and stigmasterol, $C_{36}H_{60}O_6$, are types. They have been investigated by Power and Salway (*J. Chem. Soc.*, 1913, 103, 399, 1022) who give a list of the known varieties. They are not affected by hot aqueous or dilute alcoholic hydrogen chloride, but are hydrolysed by this acid when dissolved in warm amyl alcohol. They form crystalline tetraacetyl- and tetrabenzoyl-derivatives and give the characteristic colour indication of the phytosterols when they are dissolved in acetic anhydride and chloroform and a drop of concentrated sulphuric acid is added. Sitosterol-*d*-glucoside melts at $270-300^{\circ}$; it can be prepared synthetically from sitosterol and acetobromoglucose. Glucosides of cholesterol and of fatty alcohols have been synthesised in the same manner; it is probable that they will be found in plants, (see p. 68 footnote).

Since the tannins are acyl-derivatives of glucose of the type of penta-acetyl glucose it was to be expected that simpler acyl derivatives would be found to exist naturally. The first of these to be described was *dibenzoyl glucoxylose*, a crystalline bitter substance present in the leaves and stems of *Daviesia latifolia* (*J. Chem. Soc.*, 1914, 105, 767, 1062). This is a dibenzoyl derivative ($C_{25}H_{28}O_{12}$) of a disaccharide composed of glucose and xylose, m. p. $147-148^{\circ}$. The glucoxylose has no reducing action on Fehling's solution, and therefore is of the same type as sucrose.

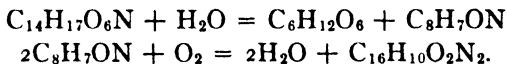
Several species of the genus *Solanum* are reported to contain bases which are both alkaloids and glucosides. *Solanguetine*, the gluco-alkaloid isolated from *Solanum angustifolium* by Tutin and Clewer has the composition $C_{33}H_{53}O_7N$, m. p. 235° (decomp.). It is hydrolysed to glucose and solangustidine and is without physiological activity. The plant also contains quercetin, rutin and *l*-asparagine. Such gluco-alkaloids are termed collectively "solanines."

Madder Glucosides

Madder is the ground root of *Rubia tinctorum*, and since ancient times has been cultivated as a dye. Its most important glucoside is *ruberythric acid*, $C_{26}H_{28}O_{14}$, which is hydrolysed by the enzyme erythrozyme to alizarin and 2 molecules of dextrose. It forms red salts. Madder also contains *purpurin*, *xanthopurpurin* (tri- and di-hydroxyanthraquinone, respectively), and *rubiadin* (a methyl xantho-purpurin united to a molecule of dextrose). See also the Tables on pp. 6-9, for the properties of these glucosides.

Indican

Indican, $C_{16}H_{17}O_6N \cdot 3H_2O$ (3- β -glucosidoxindole), is contained in indigo-yielding plants (e. g., *Isatis tinctoria*, woad) and may be extracted by means of acetone. It is of importance in that it is hydrolysed by a specific enzyme, indimulsin, to indoxyl (C_8H_7ON) and dextrose, the former being colourless but readily oxidised by a plant oxydase to the blue indigotin ($C_{16}H_{10}O_2N_2$):—



Hydrolysis is also effected slowly, but not quantitatively by emulsin, and in this case the yield of indigotin is low on account of the instability of indoxyl and its occlusion by the enzyme. In practice, the highest yields are obtained with dilute sulphuric acid in presence of a little ferric chloride to act as oxygen carrier.

The chemistry of indican has been investigated principally by Perkin and others (*J. Chem. Soc.*, 1909, **95**, 793) and by Macbeth and Pryde (*id.*, 1922, **121**, 1660). It has been synthesised by Robertson (*id.*, 1927, ii, 1937), and the m. p.'s $176-178^\circ$ and $57-58^\circ$

assigned to the anhydrous form and that containing 3 mols. of water, respectively.

The majority of tests for *indican* (e. g., in urine), involve the production of the blue indigo colour on the addition of a hydrolytic and an oxidising reagent, and extraction of the coloured substance with chloroform, (Obermayer's test, Jaffé's test etc.). Hydrochloric acid and bleaching powder are often used, but Lavalley (*Chem. Ztg.*, 1906, 30, 1251) prefers to add 3 c.c. of acid containing 5% of ferric chloride and 10 c.c. of urine, and then 3 c.c. of sulphuric acid, drop by drop. Rossi uses an equal volume of concentrated hydrochloric acid and 1 drop of a 10% solution of ammonium persulphate. Sammet, in his modification of Folin's method (*Pharm. Zentr.*, 1912, 53, 585), matches the blue colour produced in 10 c.c. of urine by 2 c.c. of a 10% solution of copper sulphate and 12 c.c. of concentrated hydrochloric acid, with that of a fresh Flehing's solution, 10 c.c. of which is taken as equivalent to an indican standard of 100, and has a colour equal to that given by a solution of 0.2228 grm. of indican per litre.

Certain substances interfere with this reaction and must be removed by precipitation of the urine with lead acetate. If iodides or skatole are present, the removal by this treatment is incomplete, and the chloroform extract should be filtered, when the indigo blue colour is adsorbed on the paper, and, unlike the starch-iodide colour which disappears, remains stable when warmed. The red deposit due to skatole sinks to the bottom of the paper cone, and the stain on the paper may be matched. Blood serum is first precipitated with an equal volume of trichloroacetic acid, filtered, and 10 c.c. of water, 1 c.c. of a 5% alcoholic solution of thymol, and 10 c.c. of reagent are added to 2.5 c.c. of filtrate. The colour is extracted and matched after 30 minutes (Snapper and Van Vloten, *Klin. Woch.*, 1922, 1, 718).

Ehrlich's Test.—The urine is boiled with an equal volume of a solution of 0.33 grm. of dimethyl-amino-formaldehyde in a mixture of 50 c.c. of water and 55 c.c. of concentrated hydrochloric acid, cooled and made ammoniacal. A red colour is produced.

The detection of indican in water is important as an indication of contamination by animal excreta. A test suggested by Jolles (*Z. physiol. Chem.*, 1915, 94, 79) depends on the fact that indican is present in urine as potassium indoxyl sulphate which is readily

oxidised by thymol and ferric chloride to 4.cymol 2.indolindolignone ($C_{19}H_{17}O_2N$; red crystals, m. p. with decomposition $218-220^\circ$), the mono-hydrochloride of which is an intense violet dye soluble in chloroform. The actual determination (*Ber. deut. pharm. Ges.*, 1920, 30, 421) is carried out on 3-4 litres of water evaporated to 250 c.c., the nitrites being removed by the addition of 3 grm. of ferrous ammonium sulphate for each 0.1 grm. per litre present. The solution is then concentrated to 10 c.c., filtered, and 1 c.c. of a 5% alcoholic solution of thymol (or α -naphthol), and 10 c.c. of fuming hydrochloric acid (sp. gr. 1.19) containing 5 grm. per litre of ferric chloride, added. The mixture is shaken for 15 minutes and then extracted with 5 c.c. portions of chloroform till no more colour is removed, the combined chloroform solutions shaken with 0.001*N* alkali, allowed to stand and then matched against a standard solution prepared in a similar fashion. The method gives the best results for dilute solutions, and is sensitive to 0.0032 mg. The violet colour is readily hydrolysed to a brown tint, which may also be matched, but this is less satisfactory (See addendum, p. 77).

Plant Pigment Glucosides

Hydroxyflavone (Anthoxanthin) Glucosides.—The majority of the soluble yellow plant pigments are salts of colourless glucosides derived from flavone or xanthone, in which the benzopyrone nucleus contains one or more hydroxyl groups, the positions of which determine the properties of the nuclear isomerides. This nucleus is united to a simple aromatic grouping (benzene or a phenol), whilst the sugar residue is usually dextrose, rhamnose or a methyl pentose. These pigments are supposed to protect the plant protoplasm from the injurious solar radiations of short wave-length, and they occur in largest quantities in plants grown in the open. They are related to the anthocyanidins (*q.v.*). A list of the most important glucosides and their principal sources follows, and further details will be found in the Tables on pp. 6-9.

Euxanthic acid (Indian Yellow), $C_{19}H_{16}O_{10}$, is an important pigment, and the principal colouring matter of this group. It is a combination of euxanthone (2.3'-dihydroxanthone) and glucuronic acid, and is found as the magnesium salt in the urine of cattle fed on mango leaves.

Apiin (parsley). *Campferitin* or *Robinin* (white azalea, Java indigo). *Fustin* (fustic). *Gentisin* (gentian). *Gossypitrin*¹ (Egyptian cotton flower). *Incarnatrin* (crimson clover) is hydrolysed by emulsin to dextrose and quercetin. *Quercetagenin* (African marigold) resembles gossypitrin. *Quercitrin*, $C_{21}H_{20}O_{11}$, (oak bark and many other sources) is used as a natural dye, and yields rhamnose and quercetin (1,3,3',4'-tetra hydroxy flavonol). It has also been said to occur in tea leaves, but the quercetin produced from them by hydrolysis may be due to rutin.

Quercimeritrin, $C_{21}H_{20}O_{12}$, (*Gossypium herbaceum*) is hydrolysed with difficulty to dextrose and quercetin, whilst its isomers *iso-quercitrin*, and *serotin* (also found in *Prunus serotina*), which are found with it, are easily hydrolysed to the same products.

Rutin (*Ruta graveolens* and *Rhamnus utilis*, etc.), $C_{27}H_{30}O_{16}$, $2H_2O$, m. p. $188-191^\circ$, is widely distributed, and has been isolated independently under the names myrticolorin, violaquercitrin, osyritin, sophorin ($C_{33}H_{42}O_{20}$, $4H_2O$) and eldrin. It occurs in the elder and probably also in tea, and is hydrolysed to dextrose, rhamnose and quercetin, (Charaux, *Bull. Soc. Chim. Biol.*, 1924, 6, 631). *Thujin* (*Thuja occidentalis*), $C_{21}H_{22}O_{11}$, m. p. $183-185^\circ$, is an isomer of quercitrin, with which it is associated, and is hydrolysed to thujigenin ($C_{14}H_{12}O_7$) and finally to thujetin ($C_{14}H_{14}O_8$), which gives a blue-green colour with alkali (Perkin, *J. Chem. Soc.*, 1914, 105, 1408).

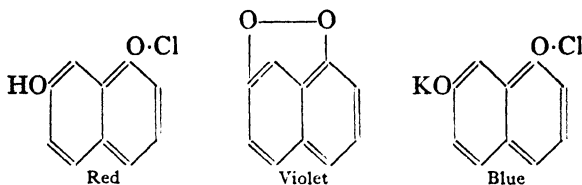
Xanthorhamnin, $C_{34}H_{42}O_{20}$, (*Rhamnus* species) yields rhamnose (2 mols.), galactose and rhamnetin (mono-methyl quercetin) and is associated with the other mono-methyl esters of quercetin.

Flavone, the parent substance, also occurs in plants in the free state and may be detected by the red colour produced on reduction of a hot alcohol extract of the plant by a drop of mercury, magnesium powder, and 3 drops of concentrated hydrochloric acid.

Anthocyanidin Glucosides

Willstätter has shown that many soluble red, violet and blue plant pigments are glucoside derivatives of the benzopyrilium type, the colours being due to the formation of salts or anhydrides with the quinonoid structures shown on p. 75.

¹ Perkin (*J. Chem. Soc.*, 1916, 109, 145) has examined the other glucosides of the cotton flowers.



They may usually be isolated by precipitation with ether of an extract of the plant in cold glacial acetic acid, followed by successive crystallisations from warm picric acid and acidic alcohol, and are best known in the form of chlorides.

Their relation to the anthoxanins is shown by the fact that products of hydrolysis of both groups are isomeric. Thus, cyanidin, pelargonidin and delphinidin are isomeric with luteolin, campferol and fisetin, apigenin and galangin, and with quercetin, respectively. They are converted into phloroglucinol when heated with alkali. The following is a list of the most important members of this group, with their principal sources, and further information may be found from the Tables on pp. 6-9.

Aceridin (*Acer plantanoides*), *cyanin* (cornflower, rose), *delphinin* (larkspur), *idain* (cranberry), *malvin* (mallow), *myrtillin* (whortleberry), *ænidin* and its isomers (blue grapes), *pæonin* (pæony), *pelargonin* (geranium). In addition, the following glucosides have been isolated by Willstätter and his co-workers (*Annalen*, 1916, **412**, 113 *et seq.*).

Ampelopsin (*Ampelopsis quinquefolia*, wild vine), $C_{22}H_{22}O_{12}$, is hydrolysed to dextrose and ampelopsidin, and gives a faint and intense violet colour with aqueous and alcoholic ferric chloride solutions, respectively.

Asterin (*Aster chinensis*) $C_{21}H_{20}O_{11}, 1\frac{1}{2} H_2O$ gives cyanidin and dextrose. *Callistephin* $C_{21}H_{20}O_{10}, 2H_2O$, an isomer of pelargonin from the same source, is soluble in 7% hydrochloric acid, and yields pelargonidin and dextrose. It gives violet and yellow-red colorations with alkali and alcohol, respectively. *Chrysanthemin* (*Chrysanthemum indicum*), $C_{21}H_{20}O_{11}$, decomposes at 205° , and is hydrolysed to dextrose and cyanidin. It differs from asterin, idain and cyanin in that it gives a cornflower blue instead of a violet colour with a solution of sodium carbonate.

Keracyanin (*Prunus avium*, cherry), $C_{27}H_{30}O_{15}$, 3-4 H_2O , yields cyanidin, dextrose and rhamnose, and gives a red-violet colour with sodium carbonate solution.

Mecocyanin (*Papaver rhæas*, poppy), $C_{27}H_{30}O_{16}$, is soluble in 10% hydrochloric acid. It is related to chrysanthemin and cyanin and gives the same reactions and hydrolysis products as the latter. *Petunin* (*Petunia*), $C_{28}H_{32}O_{17}$, $2H_2O$, m. p. 178° , yields dextrose and petunidin, and produces violet and violet-blue colours with ferric chloride in methyl alcohol and with alkali, respectively. *Prunicyanin* (*Prunus spinosa*, sloes) is amorphous, yields cyanidin, rhamnose and a hexose, and gives a blue colour with sodium carbonate or alcoholic ferric chloride solutions.

Salvianin (*Salvia coccinea*) is hydrolysed to pelargonidin, dextrose (2 mols.) and malonic acid, and, when dried, yields *salvinin*, $C_{27}H_{30}O_{16}$, (which has the same hydrolysis products and decomposes at 168°), and *salvin*, $C_{27}H_{26}O_{13}$.

Violanin (*Viola tricolor*, pansy), $C_{27}H_{28}O_{15}$, forms a number of hydrates, is hydrolysed to rhamnose, dextrose and delphinidin, and gives blue colours with alkali or ferric chloride solutions.

The determination of the partition-coefficient of an anthocyanidin pigment between 0.5% hydrochloric acid and amyl alcohol is of great analytical importance, since it is a test of its individuality and indicates whether it is a mono- or di-glucoside. A known weight of sample is shaken with equal volumes of the two solvents, and the colour produced in the alcohol layer is matched against a solution of glucoside of known strength in the same solvent. The concentration in the acid layer is then obtained by difference.

The various colours produced with ferric chloride solutions are a guide to the positions of the OH-groups. Robertson and Robinson (*Biochem. J.*, 1929, **23**, 35) have recently devised a method of identifying these glucosides based on the fact that they are indicators which yield definite colours in buffer solutions of various pH values.

ADDENDUM TO GLUCOSIDES

DIGITALIS

Biological Assay.—Further work by Trevan, Boock, Burn and Gaddum (*Quart. J. Pharm.*, 1928, **1**, 6) has shown that concordant results are obtained by their method for strong leaves, though for weak leaves it is less satisfactory. Though the International standard advocated by the Geneva Conference is a practical one for Great Britain, it is not necessarily so for other countries, and it is considered that the prohibition of deviations from standard of

more than 25% should be withdrawn. The importance of biological examination is further emphasised by Wokes (*ibid.*, 1929, 2, 48) who found that the potency of 8 samples of English digitalis leaves varied within 64-140% of their mean value which, however, was itself similar to that of the standard. The cat and frog methods give the same results on fresh samples, but after a month the latter gives consistently low values. The determination of the minimum dose to produce vomiting in pigeons has also recently been stated to give results comparable with the effects produced on human subjects.

Perrot and Bourcet (*Compt. rend.*, 1928, 186, 1021) consider that French crystalline digitalin is really digitoxin, whilst Cloetta's digitoxin is a different glucoside occurring in commercial digitalin to the extent of 20-75%. They accordingly extract 25 gm. of powdered sample, passed through a No. 30 sieve, three times with 200 c.c. of boiling 75% alcohol, filter hot, and extract the residue after evaporation with 20 c.c. of (Codex) lead acetate solution. The residue after evaporation of this extract is dried at 100°, extracted with cold chloroform for 24 hours, filtered through dry sodium carbonate and evaporated. Chlorophyll and fatty matters are removed by treatment with a mixture of 5 c.c. each of pinene and ether for 24 hours, and the residue dried, again extracted with chloroform, and the extract decolorised with 0.1 gm. of charcoal, evaporated to 10 c.c., and centrifuged with 10 c.c. of petroleum spirit (b. p. < 60°) and 10 drops of ether. The precipitate finally is dried and weighed.

A review of recent work on the digitalis glucosides is given by Windaus (*Arch. exp. Path. Pharm.*, 1928, 135, 253). See also Jacob and Gustus (*J. Biol. Chem.*, 1929, 82, 403) for the structural relationship of gitoxigenin and its *iso*-compound.

Indican in Urine

The determination of indican in urine is of special importance, since according to Mave (*Pharm. Z.*, 1928, 73, 791) it is produced in the urine by a number of drugs used in medicine, and by phenol, terpenes, and naphthalene and pyrazolone derivatives. Moreover, it may show positive reactions in certain tests for sugar. Jolles (*Arch. Pharm.*, 1928, 266, 40) has summarised a number of tests for indican in urine and blood serum.

STROPHANTHIN

Jacobs and others (*J. Biol. Chem.*, 1927, **74**, 795; 1928, **79**, 531) have examined samples of strophanthin from *S. Hispidus* and from *S. Kombé*, and conclude that though they are similar in structure, they are different substances.

U.S.P. Assay.—As a result of comparative bio-assays of tinctures of strophanthus Edmunds, Lovell and Braden (*J. Amer. Pharm. Assoc.*, 1929, **18**, 508) have concluded that the *U.S.P.* method requires revision.

SAPONINS

Sources.—Rehorst (*Ber.*, 1929, **62**, [B], 519) has recently extracted a saponin from the sugar beet in 0.5% sodium hydroxide solution. After acidification, extraction with alcohol and dialysis the compound $C_{37}H_{56}O_9$, m.p. 215–216°, $[\alpha]_D^{18} + 31.07$ (methyl alcohol) was obtained, which on hydrolysis yielded 30% of *d*-glycuronic acid and 70% of the sapogenin $C_{31}H_{48}O_3$.

Hæmolytic Method.—Additional sources of error in the hæmolytic method have been pointed out by Kofler and Adam (*Arch. Pharm.*, 1927, **265**, 624, 652) who showed that comparable results are obtained only between the same saponins and for the same blood. Even so, the *pH* value of the extraction must be adjusted to 7.4 and fresh, unwashed, cows' blood corpuscles used. They therefore extract the sample, ground to pass a No. 5 sieve, with a mixture of 0.9% sodium chloride solution and a phosphate buffer for 30 minutes on the water-bath. Sarsaparilla should, however, be extracted cold. Merck's "sap. pur. albiss," which is taken as standard, has a Kober hæmolytic index of 25,000 under these conditions. Parallel results were obtained from determinations of the strengths of lethal doses for tadpoles.

Acknowledgements are due particularly to the first-mentioned publication in the following bibliography:—

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NON-GLUCOSIDAL BITTER PRINCIPLES

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A considerable number of neutral and non-glucosidal bitter substances receive applications in medicine and as flavouring agents, etc. In many instances their chemistry has been very incompletely studied, and their feeble affinities render their available reactions few in number and wanting in precision.

The more important members of the group, *e. g.*, the aloins, santonin, colocynthin, picrotoxin, hop-bitter, and quassiin are considered in the sequel.

Bach (*J. pr. Chem.*, 1874, 117, 188; *Year-book Pharm.*, 1874, 293) published some useful hints for the detection of certain bitter principles when occurring in medicine. He pointed out that the bitter principles of aloes, colocynth, wormwood, and gentian are soluble in water, that those of agaric and scammony dissolve in ether, whilst jalap resin is insoluble in either solvent.

The scheme on the next page for the detection of the above bitter principles in medicines, etc., is a tabulated form of Bach's method of procedure.

ALOE BITTERS. ALOINS

The article known in commerce as aloes or bitter aloes is the inspissated juice which has exuded or been pressed from the leaf of various species of *Aloe*.¹ It contains a number of closely allied purgative bitter principles generically called aloins, which can be extracted from the corresponding aloes by treatment with water. The odour of aloes is due to a pale yellow, mobile essential oil, *b. p.* 266–271°, *sp. gr.* 0.863, which, when pure, has a peppermint odour. Aloes also contain salicylic and coumaric acids.

¹ Interesting information on the preparation of aloes from the juice of the plant is given in a paper by Holmes (*Pharm. J.*, 1892, 52, 233). A résumé of work on aloins is given by Léger (*Ann. Chim.*, 1917, 8, 265).

Extract the substance or residue with alcohol, and evaporate the filtered liquid to dryness. Exhaust the dried and powdered residue with cold water, and filter.		
Residue may contain resins of agaric, scammony, and jalap. Dry, and treat with ether.		
Residue consists of <i>jalap</i> resin, which gives with strong sulphuric acid a brown coloration, gradually becoming blood-red, and exhaling odour of <i>jalap</i> .	Solution. Evaporate and treat residue with warm solution of sodium carbonate.	Solution. On acidifying, <i>agaric</i> resin is precipitated, insoluble in nitric acid, but dissolved by sulphuric acid, becoming brown on heating, and decolourised by nitric acid, with separation of colourless flocks.
Residue swells to yellow mass with nitric acid. Cold sulphuric acid dissolves it with orange-red colour, changing to cherry-red, <i>scammony</i> .	Residue contains <i>colocynthis</i> , which gives a red coloration with strong sulphuric acid and a cherry-red with Frohde's reagent.	Solution, if evaporated to dryness, yields a residue which, in presence of <i>wormwood</i> , is yellowish-brown, and gives with Frohde's reagent a brown coloration, changing to green, and ultimately to violet.
Solution, concentrated at 100° if necessary, is cooled, treated with mercurous nitrate in excess, and rapidly filtered.		
Precipitate. Add ammonia in excess, filter, treat filtrate with barium acetate, and again filter.		
Filtrate is orange-red in presence of aloes. Evaporate to dryness, exhaust with alcohol, again evaporate, treat residue with nitric acid, and evaporate to dryness. If does were present, the picric acid formed will give a brown-red colour when boiled with alkali hydroxide and a reducing agent, such as potassium cyanide.		

Tilden (*Pharm. J.*, 1872, **31**, 845; 1875, **35**, 208) divided aloins into two classes, nataloins (characteristic of Natal aloes) and barbaloins (characteristic of Barbadoes aloes).

1. *Nataloins* are not reddened by treatment with nitric acid, even on heating; and do not form any definite chloro- or bromo-derivatives which yield only picric and oxalic acids as products of the oxidation.

2. *Barbaloins* are reddened by nitric acid, and in addition to picric and oxalic acids yield aloëtic acid, $C_{14}H_4(NO_2)_4O_2$, and chrysammic acid, $C_{14}H_2(NO_2)_4(OH)_2O_2$, as products of the treatment.

Shenstone (*Pharm. J.*, 1882, **42**, 461) further distinguished α -*barbaloin* from Barbadoes aloes, which is reddened in the cold by nitric acid; and β -*barbaloins* from Jafferabad, Socotrine, and Zanzibar aloes, which are not coloured in the cold by nitric acid of 1.42 sp. gr., though reddened on heating. The β -barbaloins are, however, reddened by cold *fuming* nitric acid.

According to Sommaruga (*Pharm. J.*, 1874, **34**, 23) socotrine aloes contains a mixture of an aloin of the composition $C_{15}H_{16}O_7$ with another of the composition $C_{17}H_{18}O_7$. Natal aloin is said to contain $C_{23}H_{26}O_{10}$.

Most discrepant statements are also recorded respecting the solubility, physiological activity, susceptibility to air and heat, and other characters of the aloins (see Brown, *Pharm. J.*, 1887, **46**, 678). Thus, aloin (source doubtful) is stated by various authorities to dissolve in 60, in 90, and in 500 parts of water; and to be insoluble, freely soluble, and soluble in 30 parts of alcohol.

Aloin (*aloinum*) is described in the British Pharmacopœia as small, pale, yellow, odourless, very bitter crystals almost soluble in 130 parts of water, sparingly soluble in ether, chloroform, and benzene, and completely soluble in 18 parts of 90% alcohol, ammonia or 50 parts of acetone. The tests include the Bainbridge and Morrow, and borax reactions, (p. 88), and the production of a red colour and green fluorescence in ammoniacal solutions. A solution of 0.5 grm. in 25 c.c. of warm water is filtered, and 1 drop of copper sulphate solution added to 20 c.c. of a solution of the residual pale yellow crystals in water (1 in 1000). A bright yellow colour is obtained which turns red on the addition of 0.5 c.c. of a saturated sodium chloride solution. This, in turn, becomes violet if 1 c.c. of 90% alcohol is added. The ash is not appreciable.

In addition to having these properties, the *aloinum* of the U. S. Pharmacopœia should be neutral or faintly acid, with not more than 0.5% of ash, and should give a green colour with ferric chloride solution. Only a trace of emodin (detected by Bornträger's test, p. 87) is allowed, and the insoluble residue (dried at 100°) from a solution of 1 grm. in 120 c.c. of water at 25°, shaken well for 2 hours, should not exceed 1.5%.

Aloin from Barbadoes aloes, $C_{21}H_{20}O_9$, (*Barbaloin*) was obtained by Grönewold (*Arch. Pharm.*, 1890, 228, 115) in small, pale yellow, prismatic needles, m. p. 147°, $[\alpha]_D - 10.4$ (in ethyl acetate), and -8.3 (in 90% alcohol). The air-dried crystals contain $C_{16}H_{16}O_7 + 3H_2O$, and are probably a mixture of barbaloin and *isobarbaloin*, in which the arabinose group is in the 1 and 8 position, respectively. This has been confirmed by Tschirch and Pedersen (*Arch. Pharm.*, 1898, 236, 200). $2H_2O$ are lost at 100°, and an amorphous form also exists. The moist crystals readily become discoloured, especially if exposed to light. They dissolve very sparingly in cold water, but readily on boiling, the hot solution rapidly becoming brown. Barbaloin is only slightly soluble in ether, chloroform, carbon disulphide, benzene or petroleum spirit. Acetic acid dissolves it readily, and this solution is not affected by the air. A bromaloin of the composition $C_{16}H_{13}O_7Br_3 + 4H_2O$ was described by Grönewold, who also obtained with difficulty a triacetyl-derivative, $C_{16}H_{13}O_7Ac_3 + \frac{1}{2}H_2O$, in soft yellow needles, and a hexa-acetyl-derivative forming white, hard, columnar crystals. On oxidation with chromic acid mixture barbaloin yields acetic acid, carbon dioxide, and alloxanthin, $C_{14}H_3(CH_3)(OH)_4O_2$. Seal and Kleber (*Ber.*, 1917, 50, 795) also obtained aloin emodin (trihydroxy methyl anthraquinone) as an oxidation product.

Isobarbaloin (see p. 92), occurs in aloin from Barbadoes aloes to the extent of 0.5%, but is absent from Cape or Socotrine aloes.

Grönewold regards other aloins (*e. g.* that from *Curaçao aloes*) as identical with that from Barbadoes aloes. A possible exception is Zanzibar aloes.

Aloin from Natal aloes (Nataloin) is said by Grönewold to be $C_{22}H_{23}(OCH_3)O_9$, with a variable quantity of water of crystallisation. It forms bright yellow scales or large well-formed crystals, which crystallise from alcohol, soften in a capillary tube at 180°, and melt and decompose at about 203°, and comprises 16% of the yield.

These characters, together with its resistance to the action of alkalies and the presence of a methoxyl-group, distinguish nataloin from barbaloin. On treatment with chromic acid mixture, nataloin yields acetic acid and carbon dioxide, quinol being probably formed in addition.

Aloin from Sicilian aloes (*Aloe vulgaris*) is known as *Sicaloin*, $C_{15}H_{20}O_7$. It gives no fluorescence with a solution of borax, and no Bornträger reaction (p. 87). **Aloin from Cape Aloes**:—Kiefer (*Pharm. J.*, 1925, **115**, 384) who examined the methods of the Swiss Pharmacopœia for the extraction of Cape aloes, concluded that the drug was composed of: (1) 30% each of two active, bright yellow amorphous resins; (2) 6–8% of a highly active resin, insoluble in sodium carbonate solution; (3) 5% of aloin (slightly active), (4) 1.5–1.8% of emodin (slightly active); (5) 15–20% of inactive, water-soluble substances; and (6) 5–10% of amorphous constituents, soluble in acetone, and producing abdominal pains, but with no purgative effects.

In alcoholic solution the aloins are neutral to litmus. Their aqueous solutions are coloured green or greenish-black by ferric chloride, and are gradually precipitated by a solution of basic lead acetate.

In acid or neutral solutions the aloins are tolerably stable, but in presence of alkalies, in which they dissolve with orange colour, they readily undergo decomposition.

The action of dilute mineral acids on the aloins has been studied with very discordant results. According to Czumpelik and Rochleder (1861), by treating aloin with dilute sulphuric acid, dextrose and rottlerin are produced. Kossmann describes the products of the action as dextrose, aloeresic acid, $C_{30}H_{32}O_{14}$, aloeretic acid, $C_{30}H_{34}O_{15}$, and aloeretin, $C_{30}H_{18}O_{11}$. Tilden, however, denies the formation of dextrose. Hlascwetz states that paracoumaric acid is formed, and Czumpelik and Rochleder obtained the same substance by the action of potassium hydroxide on aloin. According to Tschirch and Pedersen when barbaloin is fused with potassium hydroxide or heated with 10% sulphuric acid and steam blown through the mixture, an intensely black powder, *alonigrin*, $C_{22}H_{18}O_8$, is formed, which is soluble in alkalies, but insoluble in ordinary solvents.

According to Seel (*Arch. Pharm.*, 1919, **257**, 212 *et seq.*), the constituents of aloes of pharmacological importance are: (1) aloin

(crystalline, water-soluble), (2) aloetin (amorphous, water-soluble), (3) a resin (amorphous, insoluble in cold water), and (4) emodin (present to the extent of less than 1% and sparingly soluble in hot water). The first three constituents may be oxidised by potassium persulphate. Aloin is then converted into *puraloin I*, $C_{12}H_{10}O_6$, and then into *puraloin II*, $C_{13}H_{12}O_6$. Since these substances are stable to further oxidation and are insoluble in water or dilute acids, a quantitative method for the determination of aloes based on their production has been suggested. The latter gives a red or violet colour with alkali. Aloetin behaved similarly to aloin, but gave more secondary oxidation products, whilst oxidation of the resin by Caro's acid, showed it to be principally resinified aloin and aloetin. Aloin and aloetin are also oxidised by sodium peroxide, with the production of emodin, which is stable to further oxidation.

Great discrepancies occur in the recorded statements of the proportion of aloin present in commercial aloes. The following method, described by Tilden, is regarded by H. C. Plenge (*Pharm. J.*, 1884, **44**, 330) as the best practicable plan of preparing aloin on the small scale from most varieties of aloes: 25 grm. of the sample should be dissolved in boiling water, the liquid acidified with hydrochloric acid, and allowed to cool. It is then decanted from the precipitated resinous matter, evaporated to about 50 c.c., and set aside for two weeks for crystals to form. The liquid portion is then poured off, and the crystals pressed between folds of filter paper. The crude aloin thus obtained is contaminated with a considerable quantity of resin, from which it is best purified by treatment with ethyl acetate, with occasional agitation, till the liquid acquires a brown colour, and the yellowish colour of the crystals can be distinguished. The liquid is then quickly and carefully poured off, and the crystals dried. Treated in this manner, Barbadoes aloes, for which the method is specially adapted, gave an average of 9% of aloin; whilst Curaçao averaged 7.5, and Bonare 7%. Socotrine aloes yielded 3%, but on repeating the process on the same sample, no aloin could be obtained. It was, however, isolated to the amount of 10% by digesting 2 parts of the aloes in 3 parts of alcohol for 24 hours, and then heating the liquid over a water-bath for 2 hours. After cooling, the liquid was poured off from the resin, filtered, and set aside in a loosely covered dish to crystallise. The crystals of aloin were washed with a little alcohol, and dried.

Woodruff (*Pharm. J.*, 1889, 48, 773), recommended the following process for the preparation of aloin: 20 grm. of Barbadoes aloes were heated over the water-bath with about 40 c.c. of amyl alcohol, in a flask furnished with a reflux condenser. The liquid was decanted while hot into a beaker, where it solidified on cooling, and in 3 days formed a mass of which the major part consisted of impure crystals of aloin. The mass was pressed and percolated with carbon disulphide to dissolve admixed resin, and the solvent then washed out with benzene. From this dried crude aloin a pure product was obtained by redissolving in cold water (hot water dissolves resinous matter), filtering, and allowing the aloin to crystallise spontaneously from the filtrate. It could then be recrystallised from alcohol.

Other methods for the isolation and identification of the different aloins are given under the heading **Valuation of Aloes** (p. 91).

Serre (*Pharm. J.*, 1895, 54, 839) gives the following data illustrating the quality of typical samples of commercial aloin, which is now employed very extensively for the manufacture of pills.¹

Source	Colour	M. p.	Resin	Ash
A. American	Bright pale yellow	116°
B. American	Brown	140°	5.8%	1.4%
C. English	Greyish-yellow	145°	4.7%
		(softens only).
D. German ..	Deep bright yellow	142°	1.3%

Serre remarks that the ash of B was white, but the ash of C and of D consisted chiefly of iron, to which impurity he attributes the dull colour of sample C. He adds that the best quantitative test for resin—the precipitation of an ammoniacal alcoholic solution with a large volume of ice-water—gave negative results with C and D, although the presence of resin could be detected qualitatively, and was indicated by the colour of the preparation. Serre recommends as a test for absolute freedom from resin that 1 grm. of the finely powdered aloin should be shaken in a test-tube with 20 c.c. of water,

¹ Some useful hints on the preparation of aloin on a large scale have been published by Serre (*loc. cit.*). He points out that complete combustibility and insolubility in alcohol are no indication of the absence of resin. In preparing aloin it is usual to employ dark aloes as being of lower price, but such a practice is held by Serre to be objectionable. The selected aloes, which should be of a liver colour and clear fracture, are dissolved in water at a temperature not exceeding 40°, and, when solution appears complete, more water added as long as further precipitation of resin occurs. The liquid is then allowed to stand, and the bright solution concentrated *in vacuo* and set aside to crystallise. The mother-liquor is then drawn off, and the aloin pressed and purified by suitable solvents and recrystallisation. The last traces of resin can only be removed from the aloin by elaborate mechanical and chemical methods, impracticable on a small scale.

and allowed to stand for 1 minute. The resulting solution should be perfectly clear. A was the only one of the above samples which stood this test. A melting-point of approximately 116° should, according to Serre, be insisted on. This condition is not consistent with recorded melting points of pure aloins.

Commercial Aloes vary in physical and chemical character according to their origin. The chief varieties are those known as Barbadoes, Cape, Natal,¹ Hepatic, Socotrine, Zanzibar and Uganda² aloes. All species of aloes are official in the British Pharmacopœia (1914) which describes them as the juice which flows from the transversely cut leaves, whose common form is hard, yellow or brown masses, with fractured surfaces which are dull waxy and uniform in Curaçao and Zanzibar aloes, and even and porous in Socotrine aloes. Minute crystals are embedded in the mass, which has a characteristic odour and bitter nauseous taste. Aloes should be soluble in boiling water (0.1 grm. in 10 c.c.), and in 60% alcohol, and contain not more than 10% of ash and 5% of aloin (aloinum—see p. 81).

Cape, Curaçao and Socotrine aloes (from *A. ferox* Miller, *A. vera* L., and *A. Perryi* Baker, respectively) which are official in the U. S. Pharmacopœia, should contain not more than 4% of ash or 10% of water, and not less than 50% of water-insoluble matters. Socotrine aloes form yellow or brown, opaque, smooth glistening masses, fracturing conchoidally, Curaçao aloes are orange to dark brown, uneven, waxy and resinous, whilst Cape aloes are red-brown, with a yellow coating and a glassy fracture, and taste sour. The tests are as follows:—The powder (1 grm.) is shaken with 25 c.c. of water, filtered on a tared paper, the filtrate and washings made up to 100 c.c. with water, and the residue (which should not exceed 50%) dried over sulphuric acid and weighed. Bainbridge and Morrow's nitric acid test, Bornträger's test (p. 87) and the borax test are applied to the filtrate, which is normally yellow (Socotrine or Cape aloes) or red-brown (Curaçao aloes), but becomes dark. The absence of gummy and inorganic impurities is shown by the clear solution obtained when 1 grm. of aloes is heated gently with 50 c.c. of alcohol and cooled.

Aloes are usually compounded with myrrh, potassium carbonate and tincture of Cardamon, or with glucose syrup and asafœtida.

¹ Natal aloes have almost disappeared from commerce.

² It is said that "Uganda" aloes come not from Uganda, but from Mossel Bay in Cape Colony, (see p. 90).

The purgative effect is hastened by the presence of iron, and salts of this element are often incorporated in pills. Aloes were formerly used as a substitute for hops. They are marketed in numerous forms, such as formaloin, an insoluble and tasteless product formed by the condensation of aloes and formaldehyde in the presence of sulphuric acid (Merck). Besides the above varieties of aloes, aloes of other origin occur in commerce and a number of tests, many of them originally put forward as tests for the detection of aloes but now known to be characteristic only of aloes of particular origin, are used to distinguish between the several commercial varieties.

Bornträger's test (*Z. anal. Chem.*, 1880, **19**, 165) consists in extracting the substance with alcohol, filtering, shaking the evaporated filtrate with 2 volumes of benzene, filtering the benzene layer and agitating it with warm ammonia, when the aqueous layer will, on standing, develop a more or less pink colour. The colour is not due to aloin, but to aloë-emodin and is characteristic of hydroxy methyl anthraquinones (Tschirch and Pedersen, *Arch. Pharm.*, 1898, **236**, 200; Oesterle, *Arch. Pharm.*, 1899, **237**, 81). Though most, if not all, aloes give the reaction, it is not a safe test for aloes in admixture with other substances; but since aloes of different origin give rise to slightly different tints and intensities of colour, it is an aid in distinguishing these. Tschirch and Hoffbauer (*Schweiz. Woch. Chem. Pharm.*, 1905, **43**, 153) take 10 c.c. of a 1 in 1,000 aqueous solution for the test, shake for 1 minute with 10 c.c. of benzene, and pour the benzene into 5 c.c. of ammonia. A similar reaction is given by rhubarb and other drugs containing chrysophanic acid; hence the uselessness of the test for the detection of aloes. The difference in the intensity and shade of colour given by the several varieties of aloes is shown in the table of Cripps and Dymond on p. 89. It should be added that with some kinds of aloes a very considerable time (24 hours) is requisite for the development of the colour. If the colour disappears within 1 hour it may be due to phenolphthalein. In Lestage's modification of this test (*Ann. Chim. Anal.*, 1923, **5**, 112), 1-2 c. c. of pyridine are added to the hydrolysed sample, which is then shaken, the liquid decanted, and 4 drops of ammonia added. Rhubarb gives a pink colour and aloes a dichromate-yellow tint which changes to chromate-yellow.

Klunge's test (*Arch. Pharm.*, 1883, **221**, 363) consists in the addition, to a highly dilute and therefore nearly colourless solution of

aloes, of 1 drop of copper sulphate solution, followed by the addition of sodium chloride and alcohol. Léger (*J. Pharm. Chim.*, 1902, [vi], 15, 335) operates on a 0.5% solution of aloes made by treating with hot water, cooling and filtering from deposited resin, and to 20 c.c. of the filtrate he adds 1 drop of a saturated solution of copper sulphate, and follows this by 1 grm. of salt and 10 c.c. of 90% alcohol. Tschirch and Hoffbauer (*loc. cit.*) add 1 drop of 5% copper sulphate to 10 c.c. of a 1 in 1,000 solution of the aloes, and follow this by a trace of salt and a few drops of spirit. On the addition of the copper sulphate, the original yellow colour of the solution is intensified. The addition of the salt, however, followed by a little alcohol or warming, changes this to a red colour, the intensity and permanence of which differs with the different varieties of aloes. The use of alcohol may be avoided if the solution is warmed, but the addition of a considerable amount, as recommended by Léger, has the advantage that it dissolves the flocculent precipitate produced by the salt. The behaviour of the various sorts of aloes under this treatment is shown in the table of Cripps and Dymond, to which have been added two notes derived from Léger (*loc. cit.*).

Bainbridge and Morrow (*Pharm. J.*, 1890, 49, 570) have observed that, on addition of nitric acid to Cape aloes, a reddish colour is at first produced, but this changes in the course of 5 minutes to a green, which is permanent for several hours. This reaction (which is also known as Fluckiger's test) was always obtained with Cape aloes and was not simulated by any other variety. They applied this test to the juice of many plants of known species growing at Kew, and also showed that the deep blue colour given by **Natal** aloes is characteristic and is given by no other species. Every commercial specimen tested by these authors, however, gave some faint blue colour. The only variety of Kew aloes which gave the reaction was prepared from a specimen of *Aloes Africana*. True Socotrine aloes was found to give no reaction with nitric acid, but every commercial specimen examined gave an evanescent crimson, and the British and United States Pharmacopœias credit both Socotrine and Zanzibar aloes with the power of producing a reddish or yellowish-brown. Curaçao aloes gives a deep red colour.

Cripps and Dymond's table (*infra*, p. 89) shows the behaviour of the various sorts of aloes when subjected to the above three tests, as well as to a test devised by them. This test (*Pharm. J.*, 1885, 44,

Variety of aloes	Bornträger's test ¹	Klunge's test	Flückiger's test	Cripps and Dymond's test	
				On dilution	On adding ammonia
Barbadoes.....	Pale rose.....	Deep red ¹	Faint blue.....	Crimson.....	Deep claret.
Natal.....	Very faint pink.....	Faint red.....	Deep blue.....	Deep crimson.....	Intense brownish-red.
Curacao.....	Fine rose.....	Deep red ¹	Faint blue.....	Crimson.....	Intense claret.
Hepatic.....	Faint colour in 24 hours.....	Nil.....	Nil.....	Orange-red.....	Claret.
Hepatic (Indian).....	Faint colour in 24 hours.....	Nil.....	Nil.....	Orange-red.....	Pale claret.
Cape.....	Faint colour in 24 hours.....	Nil ²	Nil.....	Orange-red.....	Pale claret.
Socotrine (true).....	Pale rose.....	Faint red ²	Very faint blue.....	Pale crimson.....	Deep claret.
Socotrine (commercial).....	Pale rose in 24 hours.....	Nil.....	Nil.....	Orange-red.....	Claret.
Socotrine (commercial).....	Pale rose in 24 hours.....	Orange-red.....	Claret.
Socotrine (commercial).....	Pale rose in 24 hours.....	Orange-red.....	Claret.
Socotrine (Mocha or Zanzibar).....	Pale rose.....	Red.....	Faint blue.....	Crimson.....	Deep claret.
Aloes juice (Natal).....	Pale brown-pink.....	Crimson.....	Intense brown-red.

¹ Not disappearing within 12 hours (Léger: *J. Pharm. Chim.*, 1902, **15**, 335).

² According to Léger (*loc. cit.*) vinous red, fading within an hour to yellow; but Tschirch and Hoffbauer (*Schweiz. Woch. Chem. Pharm.*, 1905, **43**, 153) obtained negative results with Cape aloes.

³ If the colour fades in an hour or less, it may be due to phenolphthalein.

633) is made as follows: 1 grain of the solid substance to be tested (for this test is said to be applicable to the detection of aloes in admixture) is treated in a porcelain dish, or in a glass mortar standing on white paper, with 16 drops of strong sulphuric acid, and triturated until the whole is dissolved. Four drops of nitric acid of 1.42 sp. gr. are then added, and this is followed by 1 oz. of distilled water, when, in presence of aloes, a colour will be produced varying from deep orange to crimson, according to the kind of aloes employed. The result is confirmed by adding ammonia, when the colour is intensified, usually to a deep claret red. The test not only allows of the detection of aloes, but also gives a fair indication of the kind of aloes under examination.

Lenz extracts the solution with amyl alcohol, evaporates the extract, and adds nitric acid, potassium cyanide and potassium hydroxide solutions to the residue. A blood-red colour is produced. If 4 drops of nitric acid and 10 c.c. of water are added to a solution of 1 mg. in 16 drops of concentrated sulphuric acid, an orange or crimson colour is produced, which deepens on the addition of ammonia. This distinguishes it from that due to chrysophanic acid, which becomes pink.

Stacy (*Analyst*, 1916, 41, 75) adds a fresh solution of potassium ferrocyanide to a cold aqueous solution of aloes, when a permanent pink colour, which appears quickest in the boiling solution, is produced by 1 part in 10,000 of Barbadoes aloes. Socotrine or Cape aloes (1 part in 250) or commercial aloin give green colours. No reaction is obtained in the presence of cascara sagrada, rhubarb, acids, or large amounts of alkali, but small amounts of the latter intensify the colour, with the production of a brown tint. Previous extraction with petroleum spirit, benzene and chloroform, though not with ether or ethyl acetate, produces a negative reaction.

If the residue from an ether extract of a sample containing aloes is nitrated, reduced with stannous chloride solution at 100°, washed and 1 drop of sodium hypochlorite added, a yellow-brown solid is obtained. This test is specific for aloes.

The so-called Uganda aloes has now been known in commerce for more than 10 years. It is said not to come from Uganda at all, but from Mossel Bay in Cape Colony, and its aloin is identical with that of Cape aloes and it gives the same reactions, including the change from red to green after treatment with nitric acid (Bainbridge and

Morrow test). It contains emodin and gives a faint colour within 24 hours under Bornträger's test. (Cf. Tschirch and Klaveness, *Arch. Pharm.*, 1901, **239**, 241.)

Valuation of Aloes

From time to time proposals have been made to value aloes from the proportion of crude aloin which can be extracted under defined conditions. The method of Tilden, described under Aloins, has been used in this way.

For the estimation of aloin in aloes, Schäfer (*Pharm. Zeit.*, 1897, **42**, 95) has proposed the following method, based on the fact that aloin, in ammoniacal solution, forms compounds with the alkaline earths, which are but slightly soluble, and from which the aloin can be recovered on treatment with an acid:

50 grm. of aloes are treated with 300 c.c. of boiling water containing a few drops of hydrochloric acid, and when cold the solution is separated from the resin. Fifty c.c. of 20% ammonia and 30 c.c. of 50% calcium chloride are then added, and the whole well shaken. After 15 minutes, the precipitate is separated, and after being pressed is triturated in a mortar with a slight excess of hydrochloric acid. The aloin and calcium chloride are dissolved in as little boiling water as possible and filtered. On cooling, the aloin separates in crystals. By his method, Schäfer found from 15 to 30% of aloin in aloes of different origin.

Léger (*J. Pharm. Chim.*, 1902, [vi], **15**, 519) works on a 500-grm. sample, which he boils with a mixture of 1,800 c.c. of chloroform and 600 c.c. of methyl alcohol for 4 hours under a reflux condenser. After settling, the supernatant liquor is decanted and distilled, and the residue taken up with absolute alcohol, from which crystals of aloin separate in 3 or 4 days. By this means, 5 to 6% of aloin was obtained from Cape aloes and from Barbadoes aloes, 10% from Curaçao aloes and as much as 20% from Jafferabad aloes. Socotrine aloes gave only 4%. The method, as described, cannot be considered as possessing the attributes of a quantitative method, but Léger's object was mainly to get large quantities of the aloins to serve for their separate detection and approximate estimation. Barbaloin was identified by the characteristics of its chlor-acetyl derivative, which melts at 146.6° and forms quadratic, yellow, anhydrous crystals only slightly soluble in hot alcohol, but very soluble in

benzene. The aloin which causes West Indian aloes to give a persistent red in Klunge's test is called by L  ger *iso-barbaloin* (Compt. rend., 1914, 158, 1903). *Iso-barbaloin* can also be recognised by means of its bromine derivative, which is slightly soluble in cold alcohol, from which it crystallises in yellow needles. It gives a grey coloration with lead acetate and copper sulphate solutions which turns yellow, and when warmed with potassium chloride, deep red-violet. Barbaloin does not give this reaction. Socotrine aloes were found to consist almost exclusively of barbaloin, with a minute quantity of *iso-barbaloin*. Cape aloes were entirely free from *iso-barbaloin*, whilst Cura  o aloes contained the two aloins in approximately equal proportions. The aloin of Jafferabad aloes is mainly *iso-barbaloin*. Natal aloes contains neither barbaloin nor *iso-barbaloin*, but two distinct aloins to which are due the characteristic reaction with sulphuric acid and with the vapour of nitric acid.

Aloin is not the only active constituent of aloes, aloec-emodin having marked purgative properties; and Tschirch and Hoffbauer (*Schweiz. Woch. Chem. Pharm.*, 1905, 43, 153, Abs., *Chem. Zeit. Rep.*, 1905, 29, 106) propose to estimate the active constituents of aloes as follows: 5 grm. of the sample are digested in a 50 c.c. flask with 5 c.c. of methyl alcohol for 2 hours. The liquid is then warmed to 50 to 60  , 30 c.c. chloroform added gradually with continuous stirring, and the mixture allowed to stand for 30 minutes. The yellow-coloured chloroform solution is then filtered from the separated resin, into a tared flask, and the chloroform distilled off and used again to extract the aloin remaining with the resin, etc., in the first flask. The second chloroform extract is filtered into the tared flask, and the chloroform distilled off and again used to extract the residue in the first flask, this procedure being repeated four times in all. When the chloroform has been distilled off for the last time, not less than 4 grm. of extractive matter should be found in the tared flask. The residue in the original flask is regarded as valueless resin and the authors found 63% of such "resin" in Socotrine aloes, 33% as the average for Cura  o, and only 13 to 19% in Cape aloes, which they prefer.¹

L. v. Itallie (*Pharm. Weekblad*, 1905, 42, 553; Abs. *Pharm. J.*, 1905, 75, 554) considers the above method open to objection on the ground

¹ Other continental authorities express a preference for Cape aloes, but in Great Britain their chief use is said to be in veterinary practice. See Seel, p. 83.

that much is left behind and counted as resin which is not resin at all, but other matters protected from the solvent action of the chloroform by a coating of resin. He recommends the following modification: 5 grm. of the powdered sample are warmed with 5 c.c. of methyl alcohol in a 50 c.c. flask until a homogeneous liquid is obtained. 30 c.c. of chloroform are then added, and the mixture violently and continuously shaken for 5 minutes. By this time most of the separated resin will have adhered to the sides of the flask. After standing till clear, the liquid is decanted. The residue is again dissolved in methyl alcohol, and the resin again precipitated by means of chloroform, and this treatment is repeated once more. In this way v. Itallie found 18 to 43% of resin in Cape aloes, and only 11 to 21% in Curaçao aloes. The same author attempted the estimation of aloin by precipitation as tribromoaloin. With pure aloin the method was successful, but, applied to aloes from the Cape and Curaçao, it indicated over 70% of aloin, which v. Itallie considers to be much too high.

Detection of Aloes¹

The aqueous solution of aloes is coloured dark brown by alkalies, and olive-green or greenish-black by ferric chloride.

According to W. Lenz, if aloes be extracted with amyl alcohol, the solution evaporated, the residue treated with nitric acid and again evaporated, and this second residue boiled with potassium hydroxide and potassium cyanide, a blood-red coloration will be obtained. This test is evidently based on the formation of picric acid by the action of nitric acid on aloin and its subsequent reduction to picramic acid by treatment with potassium cyanide.

Lenz also found that useful reactions for aloes are obtained by treating an aqueous solution of the amyl alcohol extract with basic lead acetate, mercurous nitrate, tannin, and brominated potassium bromide. The extract reduces gold chloride and alkaline cupric solutions.

Weak (0.1% to 1%) aqueous solutions of aloes develop a green fluorescence on the addition of 5% of their weight of powdered borax.

For the detection of aloes in pharmaceutical preparations, Léger (*J. Pharm. Chim.*, 1902, **15**, 335) treats 1 grm. of the sample with 100 c.c. of hot water, cools rapidly, and filters from deposited resin,

¹ See also the Pharmacopœia tests on p. 81.

the filtration being accelerated by the addition of a little powdered talc. A few decigrams of sodium peroxide are introduced, little by little, into 20 c.c. of the filtrate, previously heated to 90°. An immediate evolution of oxygen occurs, and the liquid becomes brown, subsequently changing to bright cherry-red on further addition of peroxide, if aloes is present. The reaction is said to be due to the aloë-emodin of Tschirch and Oesterle (*Arch. Pharm.*, 1898, **236**, 205; 1899, **237**, 81) and, like Bornträger's reaction, is given by other drugs, such as rhubarb, which contain chrysophanic acid (dihydroxymethylanthraquinone), an emodin (trihydroxymethylanthraquinone) or rhein (tetrahydroxymethylanthraquinone), which dissolve in sufficient quantity to falsify the results. The presence of hydroxymethylanthraquinones can be detected by adding a small quantity of sodium hydroxide solution, with which they give an immediate red coloration, whilst the solution of pure aloes remains yellow. If hydroxymethylanthraquinones are detected in this way, they may be removed from the bulk of the solution to be tested in either of two ways. Either they may be precipitated by addition of a slight excess of basic lead acetate, (the method preferred by Léger), or they may be removed as follows: To 50 c.c. of the original 1% solution to be tested, 20 c.c. of 5% alum solution are added, and this is followed by excess of ammonia and finally by acetic acid until the liquid is just acid. The mixture is then filtered, and 20 c.c. of the clear filtrate tested with sodium peroxide as described. In the presence of aloes a distinct reaction will be given, whilst rhubarb extract treated in the same way gives only a faint peach coloration.

The following method due to Mossler (*Pharm. Post.*, 1913, **46**, 313, 325) will detect as little as 0.2 grm. of aloes extract in 5 grm. of a mixture of extracts of rhubarb, frangula and cascara sagrada. The alcoholic extract is evaporated to expel the alcohol, the residue taken up in water, and the liquid filtered. The filtrate (100 c.c.) is heated for 30 minutes on the water-bath with 5 c.c. of 10% sulphuric acid, the sulphuric acid precipitated with the exact amount of barium hydroxide solution, and the filtrate from the barium precipitate concentrated to 100 c.c. and clarified with lead acetate solution, care being taken to avoid a large excess. Of the solution, 10 c.c. are filtered, freed from lead by means of sodium sulphate solution or dilute sulphuric acid, and divided into two portions. One of these is

shaken with 5 c.c. of benzene, and the benzene extract shaken with dilute ammonia. If the hydroxymethylantraquinones have been completely precipitated, the aqueous layer should not show more than a light rose colour, whilst a yellow coloration of the benzene layer indicates aloes. If, however, the aqueous layer is distinctly red, the bulk of the original solution must again be treated with lead acetate, after which 10 c.c. are filtered, freed from lead, divided into two portions, and one of these portions tested with benzene and ammonia as before. When the precipitation of the hydroxymethylantraquinones is shown to be complete, the other portion of the filtrate is treated with an excess of bromine water, which, in presence of aloin, gives an immediate flocculent precipitate. The main solution is now freed from lead and 10 c.c. portions of the filtrate are tested for the usual reactions of aloes.

For the identification of the different hydroxymethylantraquinone drugs in admixture with aloes an alcoholic extract is brought to a strength of 50% of alcohol and filtered. The filtrate is boiled for 30 minutes beneath a reflux condenser with about 5% of sulphuric acid, cooled and filtered, and the alcohol expelled from the filtrate by repeated evaporation with water. The hydroxymethylantraquinones are precipitated, whilst aloin remains in solution. The precipitate is washed free from acid, dried, boiled with benzene, and the benzene extract shaken with 10% sodium carbonate solution and then with dilute sodium hydroxide solution. The two alkaline extracts are separately acidified with hydrochloric acid, each shaken with 20 c.c. of benzene, and 5 c.c. of each benzene extract are evaporated. The residues are heated with 3-4 drops of acetic acid, and the solutions transferred to glass slips and examined, after 30 minutes, in polarised light. The crystals thus obtained from rhubarb, cascara sagrada, senna, etc., show pronounced differences, as is also the case with crystals obtained by sublimation.

Hubbard (*J. Ind. Eng. Chem.*, 1917, **9**, 518) applies the following colour tests:—

	Ammonium thiocyanate	Ammonium molybdate	Borax	Uranium acetate
Senna.....	Yellow-brown.	Yellow-brown.	Brown or nothing.
Rhubarb.....	Yellow.	Mahogany.	Rose.	Mahogany-red.
Cascara.....	Red-brown.	Brown.
Aloes.....	Red in ether- layer, brown in aqueous layer.	Green.

Rhubarb only, gives red and blue colours with bleaching powder and ferrous sulphate solutions, respectively, and senna is best detected by elimination, though it sometimes gives a doubtful Bornträger reaction.

Beal and Okey (*J. Amer. Chem. Soc.*, 1917, **39**, 716) shake a cooled extract of the drug in hot 50% alcohol four times with benzene and 30% sodium hydroxide solution. A permanent light red to deep violet colour denotes cascara, rumex, rhubarb, frangula, senna or aloes. The residue from the evaporation of another benzene extract is orange-red, and turns purple-red on the addition of a solution of potassium cyanide in potassium hydroxide solution. Aloes may then be identified by eliminating tests. Thus, when the benzene extract is re-extracted with amyl alcohol the new extract gives a deep red colour and a dark green fluorescence with concentrated ammonia if aloes or cascara, or both, are present, and a red colour with mercurous nitrate in the presence of the former only. Confirmatory tests may then be applied.

Cripps and Dymond applied their test (p. 89) to various complex mixtures, and found that aloes could always be detected when present and, of the numerous pharmaceutical preparations tried, no other substance gave a similar reaction except senna, and substances, such as rhubarb, which contained chrysophanic acid. But a nearly colourless aqueous solution of aloes is not changed by ammonia, whereas with rhubarb and other substances containing chrysophanic acid a pink colour is developed. An ethyl acetate extract of rhubarb is coloured deep red on treatment with strong sulphuric acid, as is a similar extract of aloes. But on subsequently adding nitric acid the colour due to aloes is intensified, whereas that produced by

chrysophanic acid is immediately destroyed. This enables aloes to be detected in the presence of rhubarb, etc., whilst the pink colour produced in an aqueous extract on addition of ammonia is an indication of the latter and is obtained in the presence of the former.

For the estimation of aloes in mixtures, H. Hager evaporates the liquid to dryness, and macerates the cooled and pulverised residue with a mixture of 2 vols. of chloroform, 3 of benzene, and 1 of absolute alcohol. This dissolves the resins of jalap, scammony, myrrh, senna, guaiacum, etc. The residue, which contains the aloes intact, is treated at 50° with 80% alcohol. The solution is evaporated to dryness in a weighed dish, and the residue treated with 12 to 15 c.c. of a 2% solution of ammonia for every grm. of residue. To the filtered liquid an excess of lead acetate is added, and a few drops of ammonia to ensure an alkaline reaction. The precipitate, which contains all the aloes, is washed with water and mixed with ammonium sulphate. The mixture is then exhausted with 80% alcohol, and the weight of aloes ascertained from that of the residue left on evaporating the filtered alcoholic solution.

For the detection of aloes in animal matters, such as fæces, J. Dietrich (*Dorpat Thesis*, 1885; abst. *Analyst*, 1885, 10, 186) digests the substance with water acidified with sulphuric acid, then macerates for 12 hours with 3 vols. of strong alcohol, concentrates the filtered liquid, and agitates the residue successively with petroleum spirit and amyl alcohol. On evaporating the latter liquid, the aloin is obtained in a state fit for the application of the ordinary tests. Dietrich treats it with nitric acid, evaporates at 100°, dissolves the residue in alcohol, and treats the deep red solution with a drop of an alcoholic solution of potassium cyanide, which produces a rose coloration in presence of aloin. Dietrich is of opinion that the greater portion of aloes or aloin taken is excreted with the fæces; a small portion only is absorbed and passes mostly through the kidneys, whilst the remainder enters the liver, and is conveyed with the bile back into the intestines.

Artemisia Bitters

Various species of *Artemisia* contain non-glucosidal bitter principles, of which santonin is the most important. Absinthiin is a bitter principle contained in wormwood.

Other constituents of *santonica* are 1-2% of an oil, b. p. 250° (approx.), and containing α -pinene, terpinene, terpineol, terpinenol and a sesquiterpene, and *artemisin*, $C_{15}H_{18}O_4$, (m. p. 200°), which is found in the mother-liquors after the extraction of *santonin*. It crystallises from chloroform with 1 mol. of this solvent, turns yellow in the air, and is more soluble in alcohol or water than *santonin*, of which it is probably an hydroxy derivative, (Bertolo, *Gazzetta*, 1920, 50, 109).

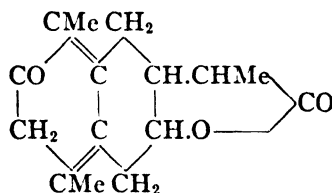
A. brevifolia has been found to contain *l*-camphor (m. p. 177° , $[\alpha]_D - 44$), a terpene essential oil (b. p. $110-180^{\circ}$, d. 0.950), and 0.1% of *brevifolin*, an optically inactive crystalline substance (m. p. 80°) which is unaffected by light and gives a blue colour with concentrated nitric acid (*Pharm. J.*, 1927, 119, 688).

Santonin, $C_{15}H_{18}O_3$, is commonly obtained from *Santonica*, also called "worm-seeds" (*Flores cinæ*). This consists of the unexpanded flower heads of *Artemisia maritima*, *A. cinæ*, *A. brevifolia*, or of closely allied varieties, (particularly those found in America and India) which contain from 1-3% of the bitter principle (see Viehovever and Capen, *J. Amer. Chem. Soc.*, 1923, 45, 1941, and *Bull. Imp. Inst.*, 1923, 21, 316).

The leaves of genuine commercial *santonica* are linear-lanceolate, with a rounded apex and an apiculus mid-rib, with many branches connecting it with two marginal veins running parallel to the margin at about one third of the distance between it and the mid-rib. There are many sessile glands, but no long apical marginal hairs, and 14 to 20 (usually 16) flower head bracts. The mid-rib branches freely in contorted veinlets (often anastomose), and the apices of the corolla lobes are only slightly papillose and bear no trichomes. The absence of *santonin* is characterised by hairy leaves and apical marginal hairs on the bracts of the flower heads. *Santonin* may be detected in the plant by extracting the flower-heads with benzene, evaporating the extract, and heating the residue to $150-170^{\circ}$. The sublimate is collected on cooled glass cover-plates, and the crystals may be identified by the tests given below, or by the production of green-brown plates (m. p. 113°) on the addition of a solution of iodine in hydriodic acid. Another micro-test consists in the striations of carmine red produced on the crystals when these are warmed gently with a little powdered potassium hydroxide on an inclined microscope slide.

Santonin is the anhydride or lactone of santoninic acid, $C_{15}H_{20}O_4$, a derivative of naphthalene.¹

A very large number of derivatives of santonin have been studied, especially by Cannizzaro, Gucci, Grassi-Cristaldi, Andreocci, Francesconi, Montemartini and Wedekind. For a convenient summary of this work see the Monograph "Die Santoningruppe" by E. Wedekind in Ahren's "Sammlung chemischer und chemisch-technischer Vorträge," 1903. Several subsequent papers have appeared, more particularly by Wedekind and Francesconi and Cusmano. The chemistry of this group is not yet fully worked out, but santonin is probably



Santonin crystallises in flattened columns, in feathery radiating groups, or in pearly plates having a slightly bitter after-taste. It has sp. gr. 1.1866, melts at 169 to 171° (Menge, *U. S. Hygienic Lab. Bulletin*, 70), and when heated cautiously may be sublimed

¹ For the preparation of santonin from worm-seeds, the volatile oil is first extracted with petroleum spirit, and 200 grm. of the residue then boiled with 70 grm. of slaked lime, 400 c.c. of water, and 400 c.c. of rectified spirit, this treatment being twice repeated. The solution of calcium santoninate is filtered, evaporated to about 500 c.c., and a little hydrochloric acid added. The greenish-brown resin thereby separated is filtered off, and the filtrate treated with a slight excess of hydrochloric acid. This liberates santoninic acid, which soon decomposes with formation of santonin, which crystallises out. The product is purified by washing with cold water, solution in alcohol, decolorisation with animal charcoal, and recrystallisation. This must be carried out in the dark, or the yellow *photo-santoninic acid*, $C_{15}H_{18}O_4$, may be produced.

The manufacture of santonin on a large scale is carried out at Tschimkent, which is conveniently situated near to the principal source of the plant on the Kirghiz steppes. The worm-seeds are ground with lime and water, hot alcohol added to the cooled mixture, the alcohol distilled off, and the residual liquid neutralised with hydrochloric acid. The crude santonin, which crystallises out after a few days, is washed with cold water. During the process of extraction a quantity of resinous substance is formed, which on treatment with a warm solution of sodium carbonate yields a large quantity of santonin. The animal charcoal which is employed to decolorise the alcoholic solution absorbs a large quantity of santonin. The yield of pure santonin obtained amounts to 1.8 to 2.0% of the plant originally taken. A. Busch recommends the separation of the accompanying resin from crude santonin by the addition of lead acetate to the alcoholic solution (*J. prakt. Chem.*, 1887, 35, 322; abst. *J. Soc. Chem. Ind.*, 1887, 6, 559). According to a modified process, the worm-seed is treated with milk of lime, the extract precipitated by sodium carbonate, and the filtered liquid warmed to 70° and decomposed with sulphuric acid. On cooling, santonin separates in large crystals.

To test the solutions, etc., obtained in the process of extraction for santonin, J. Kossowski precipitates any colouring matter with basic lead acetate, and gently heats a few drops of the filtrate in a porcelain crucible. Concentrated sulphuric acid is then added, and on further heating produces a violet coloration, the intensity of which is an indication of the amount of santonin present (*Dingl. polyt. Jour.*, 1888, 268, 42; abst. *J. Soc. Chem. Ind.*, 1888, 7, 422, 458).

unchanged. When more strongly heated santonin becomes reddish-brown, evolves white fumes, and on cooling sets to a clear brown vitreous mass, which is reddened on treatment with a little dry alkali hydroxide or slaked lime.

On exposure to light, especially to direct sun-light, santonin acquires a yellow colour. The hot alcoholic solution of this altered substance is yellow, but deposits crystals of colourless santonin on cooling.

Santonin is very sparingly soluble in cold water (1:5,000 at 15°), but dissolves in 250 parts of boiling water. One grm. is soluble in 43 c.c. of cold rectified spirit, and in 6.5 c.c. at the boiling-point; in 110 c.c. of cold or in 42 c.c. of boiling ether, and in 1.7 c.c. of chloroform, (U. S. Pharmacopœia). The solution of santonin in boiling water is scarcely perceptibly bitter, but the cold alcoholic solution has an extremely bitter taste, and is lævorotatory. For an alcoholic solution $[\alpha]_D = -173.8$; in chloroform $[\alpha]_D = -171.4$. The concentration has practically no effect on the specific rotation. Solutions of santonin do not redden litmus, but the solid substance dissolves readily in alkaline liquids to form santoninates. On adding excess of hydrochloric acid to the alkaline solution, and immediately shaking the milky liquid with ether, santonic acid is extracted.

Santoninic acid, $C_{15}H_{20}O_4$, rapidly separates from its ethereal solution in granules, and by recrystallisation from alcohol is obtained in fine rhombic crystals. It does not become yellow on exposure to light, and is very sparingly soluble in cold, but more readily in hot, water. The solution has an acid reaction and is lævorotatory, ($[\alpha]_D = -25.8$ in 97% alcohol, $c = 1 - 3$). When heated for some time at 120° santonic acid decomposes into santonin and water, and the same reaction occurs more readily on addition of a mineral acid to its solution, especially on warming.

Sodium santoninate, $C_{15}H_{19}O_4Na + 3H_2O$, forms colourless rhombic crystals, soluble in 3 parts of cold water, and also soluble in alcohol.

Calcium santoninate, formed in the extraction of santonin from worm-seed, crystallises in colourless silky needles, soluble in water and in dilute spirit, but almost wholly insoluble in absolute alcohol. The salt is not decomposed by carbon dioxide, but stronger acids cause a separation of santonin.

Santoninates of the heavy metals can be obtained by precipitation. When boiled with water such a santoninate is decomposed into pure santonin and the corresponding metallic oxide.

On boiling santonin for 12 hours with strong baryta water, acidifying the liquid with hydrochloric acid, and shaking with ether, *santoninic acid*, $C_{18}H_{20}O_4$, is extracted. This substance is more stable than its isomer, *santoninic acid*. It forms rhombic crystals which melt at 161 to 163° , is unaffected by light, and dissolves sparingly in water but readily in alcohol, to form lævorotatory solutions ($[\alpha]_D = -74^\circ$ in chloroform).

When treated with excess of mineral acids, santonin forms *santonin resin*. This product is obtained most readily by heating santonin with concentrated hydrochloric acid under pressure. It is formed during the manufacture of santonin, and appears to be a mixture of products of its decomposition.

When solid santonin is agitated with a 5% solution of potassium hydroxide in alcohol it dissolves, with transitory carmine-red coloration. The test may be modified by moistening a mixture of equal parts of slaked lime and sodium carbonate with alcohol, and adding santonin, when a fugitive purple-red coloration is developed.

Pain (*Pharm. J.*, 1901, 67, 131) has improved on this test as follows: A few crystals of santonin (1 mg. serves) are warmed with 2 to 3 c.c. of ethyl nitrite solution (British Pharmacopœia). Unlike aloin, resorcinol and many other substances, santonin gives no red colour with ethyl nitrite alone, but, on addition of a few drops of potassium hydroxide solution, a fine rose-red colour is produced. Thymol gives a dark yellow colour if present in a solution tested in this manner.

According to A. Busch, dextrose is a product of the action of dilute sulphuric acid on santonin.

If santonin is dissolved in slightly diluted sulphuric acid, the solution warmed on the water-bath, and a few drops of ferric chloride solution added, a red ring, changing to purple, is developed round each drop of the reagent, and on continuing the heating the purple colour changes to brown. T. Salzer applies the test by treating 0.01 grm. of santonin with 1 c.c. of strong sulphuric acid and 1 c.c. of water. This mixture acquires a yellow colour, and on then adding ferric chloride and warming the violet coloration is produced. The red colour may be extracted in amyl alcohol.

Reichard (*Pharm. Zeit.*, 1907, 52, 115) gives the following reactions of santonin:—A mixture of santonin with ammoniated mercury or with mercurous nitrate is coloured black on adding sulphuric acid. A mixture of santonin with cupric sulphate or chloride, when treated with sulphuric acid, gives a fine blue colour. Bismuth subnitrate and santonin also give a deep blue colour when touched with sulphuric acid. Diphenylamine, santonin and sulphuric acid do not interact when cold, but, on warming, the mixture becomes intense red; the colour changes to yellow after about 12 hours and afterwards becomes dark green. Cold sulphuric acid alone does not give a coloration with santonin, but, on warming, an intense blue colour is developed, which on further warming becomes yellow.

When santonin is treated with dilute phosphoric acid, and the solution evaporated at 100°, a yellow coloration is produced, subsequently changing to purple-red, whilst Ekkert (*Pharm. Zentr.*, 1927, 68, 545) gives a range of colour changes from red to blue produced on the addition to 1 and 2 c.c. of a 1% alcoholic solution of santonin and 4 drops of 1% furfuraldehyde, of 0.5–3 and 1–6 c.c. of concentrated sulphuric acid. A 1% solution of dextrose or of lævulose, in place of furfuraldehyde gives a garnet-brown tint.

If a solution of santonin in strong sulphuric acid is warmed on the water-bath for a few hours, and then diluted with water, *iso-santonin*, $C_{15}H_{18}O_3$, isomeric with santonin itself, is precipitated. When recrystallised from boiling alcohol this melts at 138°. It also differs from santonin in being unaltered by exposure to light, and in giving no red coloration with sulphuric or phosphoric acid.

The tentative method of the A.O.A.C. (See under Glucosides, p. 15) is adapted from Caspari's modification of Fromme's method (*Amer. Pharm. Assoc.*, 1914, 634). Ten grms. of the sample ground to pass a 30 mesh sieve are extracted in a Soxhlet apparatus for 3 hours with chloroform. All but 8 c.c. are distilled off, 100 c.c. of a 5% solution of barium hydroxide added, and the remaining solvent removed on the water-bath. The solution is then boiled, cooled, saturated with carbon dioxide (previously bubbled through sodium bicarbonate solution to remove acid), filtered on a Buchner funnel, and the residue washed twice with 10 c.c. of water. The filtrate is heated on the water-bath for 5 minutes with 5 c.c. of dilute hydrochloric acid (2 + 1), cooled, and extracted successively with 20 c.c. and two 15 c.c. portions of chloroform. The residue, left on filtration and

evaporation of the total extract, is dissolved in 9.5 c.c. of warm absolute alcohol in a stoppered flask, and a minute crystal of santonin added, (or the side of the flask scratched with a rod) to induce crystallisation. After 24 hours at 15–17°, the crystals are filtered off, washed twice with 10 c.c. of 15% (by weight) of alcohol at 16°, the crystallising flask and filter-paper dried at 100°, and a solution of the crystalline santonin in chloroform transferred to the flask. The solvent is removed, and the santonin dried at 100° and weighed, 0.04 gm. being added to the weight found to correct for solubility in dilute alcohol. Vogtherr (*Arch. Pharm.*, 1926, **264**, 324) and Kariyone and Kimura (*J. Pharm. Soc. Japan.*, 1920, 927) describe other variations of Fromme's method.

Van den Berg (*Pharm. Weekblad*, 1923, **60**, 858) extracts a suspension of the powder (5 gm.) in 30 c.c. of hot water with 10 c.c. of hydrochloric acid and 60 c.c. of chloroform, in the presence of 5 gm. of tragacanth. The extract is concentrated and boiled for 10 minutes under a reflux condenser with 50 c.c. of 15% alcohol, filtered, the paper (which is tared) washed twice with 10 c.c. of boiling dilute alcohol, and the filtrate and washings weighed after 24 hours. The flask and paper are then dried at 100–105° and weighed, and 6 mg. added to the weight for every 10 gm. of liquid in the flask before filtration.

For rapid working the method of Eder and Schneiter (*Analyst*, 1927, **52**, 40) is to be recommended. The fine powder (10 gm.) is shaken for 30 minutes with 100 gm. of benzene, the mixture filtered through an 18 cm. paper, 81 c.c. of the filtrate evaporated, and the residue heated under a reflux condenser with 40 c.c. of 15% (by weight) alcohol for 15 minutes. The hot solution is filtered through wool, boiled with 0.1 gm. of kaolin for 15 minutes under a reflux condenser, and filtered into a tared flask. After 24 hours in the dark the santonin will have crystallised, and the flask and contents are weighed. The crystals are then filtered off and weighed in the usual way, and the usual solubility corrections made. It is advisable to add a known amount (about 2%) of santonin to the santonica before making the determination, if proof of its complete absence is required.

In the above methods the presence of resins may cause low results, and the procedure of Schaap (*Pharm. Weekblad*, 1924, **61**, 277), based on that of François (*J. Pharm. Chim.*, 1922, **26**, 339), should

therefore be mentioned. The dried powder (5 gm.) is made into a paste with 1.5 gm. of slaked lime and water, boiled for 30 minutes with 100 c.c. of water, a concentrated solution of 5.5 gm. of zinc sulphate added, and the boiling continued for 15 minutes. The liquid is filtered, and the residue is re-boiled with water, and re-filtered, and the combined filtrates (zinc santoninate) evaporated with 3 c.c. of 30% nitric acid. The residue is again evaporated with 25 c.c. of water, and again with 10 c.c. of water, 2 gm. of calcium carbonate and a little sand. It is then extracted with 50 c.c. of chloroform, filtered after 2 minutes, 40 c.c. of the extract evaporated, the residue dissolved in 5 c.c. of methyl alcohol, and the solution diluted with 35 c.c. of water at 60°. After 24 hours in the dark the crystals of santonin are filtered off on a tared paper, washed 5 times with 2 c.c. of water, dried at 100° and weighed. François (*loc. cit.*) heats the mixture of lime and santonica under a reflux condenser for 1 hour with 50 c.c. of 95% alcohol at a temperature just below the b. p. This is repeated 3 times, and the filtrate from the combined extracts allowed to cool, so that, if the drug is made up with cacao butter, the fat may be separated.

When taken internally, santonin produces a remarkable effect on the vision, all objects appearing at first of a bluish tint, but subsequently yellow or greenish-yellow.¹ The taste and smell are also affected in some instances. In larger doses, santonin produces marked poisonous effects, the principal symptoms being headache, giddiness, shivering, stertorous breathing, followed by tetanus, diminished action of the heart, convulsions, and finally death by asphyxia. The pupils are dilated, and vomiting occurs in some cases. The *post-mortem* appearances are not characteristic.

It is considered that the lactonic properties of santonin cause it to act directly on muscular tissue without intervention of the nervous system, and a method of biological assay based on this fact has been suggested by Schneider (*J. Pharm. Chim.*, 1928, 8, 378) in which the increase in the number of contractions per minute of living earth-worms when transferred from a standard calcium chloride solution to the santonin solution is determined. The absence of

¹ This curious and characteristic effect is apparently due to a direct action of santonin on the nervous elements of the retina, rendering the eye less sensitive to the rays of high refrangibility (small wave-length). The eye thus becomes colour-blind to the violet end of the spectrum. Hence only the less refrangible rays produce a visual impression, and all objects appear yellow.

other substances (e.g., coumarin) which produce a like effect, must however, be ensured.

For the detection of santonin in urine, Daclin (*J. Pharm. Chim.*, 1897, 6, 534) treats 30 to 40 c.c. with lead acetate and then with solid sodium sulphate and filters. The filtrate is divided into two portions, each of which is evaporated in a porcelain basin. The residue in one is gently warmed with 1 or 2 drops of sulphuric acid, when, if santonin is present, an immediate violet coloration appears. The other residue is tested with alcoholic potassium hydroxide. Or urine may be extracted with chloroform, the latter evaporated, and the residue tested as described. Santonin imparts a saffron-yellow colour to urine, which turns violet on the addition of alkali.

Santonin (*santoninum*) has now replaced santonica in the British and U. S. Pharmacopœias, and in the former case must be derived from the unexpanded flower heads of *A. maritima*. It forms neutral, colourless, flat rhombic prisms, m. p. 170° , having a slightly bitter after-taste, no appreciable ash, and soluble in 2.5 parts of chloroform and 50 parts of 90% alcohol, but not in dilute mineral acids. It is almost insoluble in cold water, and slightly soluble in boiling water. The U. S. Pharmacopœia requires that the ash shall not exceed 0.1%, and a solution of 0.1 gm. in 2 c.c. of sulphuric acid shall give only a pale yellow colour. If to 10 c.c. of a solution of 0.5 gm. in 20 c.c. of water and 2 c.c. of dilute sulphuric acid, boiled, cooled and filtered, are added 10 c.c. of water and some iodine, no turbidity should appear after 3 hours. This last test, which denotes the absence of alkaloids, is of importance, since accidents have arisen through contamination of santonin with strychnine. A similar precaution is given in the German Pharmacopœia.

Absinthiin, $C_{15}H_{20}O_4$, is a glucosidal bitter principle existing in wormwood, *Artemisia absinthium*, wherein it is associated with an essential oil, to which is ascribable the toxic action sometimes observed as the result of absinthe drinking. The tonic effects of wormwood are due to the absinthiin.

For the preparation of absinthiin, the plant is exhausted with ether, the ether distilled off, and the residue taken up with water. The filtered liquid is shaken with hydrated alumina, again filtered, and evaporated *in vacuo*; or shaken with ether and the ether evaporated. Absinthiin thus obtained is described by O. Senger (*Arch. Pharm.*, 1892, 230, 94) as a yellowish, vitreous, intensely bitter

substance, melting at 65° . This probably contained resinous impurities, since pure absinthiin crystallises in fine white prismatic needles, m. p. 68° . It is soluble in cold water, alcohol, chloroform, benzene and ether, but only sparingly soluble in petroleum spirit and hot water. When boiled with dilute sulphuric acid, absinthiin yields dextrose, a volatile product which appears to be an ethereal oil, and an amorphous resinous compound of the formula $C_{21}H_{26}O_6$, which is apparently a hydroxy acid of the aromatic series. It yields phloroglucinol by the action of alkali hydroxides, while formic, acetic, and propionic acids are among the products of its oxidation by chromic acid mixture. When oxidised with concentrated nitric acid or potassium dichromate, absinthiin yields oxalic and picric acids. It is precipitated by tannic acid, reduces warm gold chloride solution, and gives a brown colour with Fröhde's reagent or sulphuric acid, which change through violet and green, respectively, to blue.

Colocynth Bitter

The fruit of the seed-free colocynth or bitter apple, *Citrullus colocynthis*, was originally supposed to contain a neutral bitter principle called *colocynthin*. This was extracted from bitter apples by exhausting the fruit with alcohol, evaporating the tincture to dryness, taking up the residue with water, precipitating the liquid with lead acetate, removing the excess of lead from the filtrate, and precipitating the colocynth with tannin. The compound with tannin was then decomposed by treatment with lead carbonate, and the colocynthin dissolved out by boiling alcohol.

Colocynthin was described as forming a yellowish powder or microscopic crystals, intensely bitter, and poisonous, and readily soluble in water and in boiling alcohol to form solutions neutral to litmus. The alcoholic solution was precipitated on addition of ether.

According to Walz, colocynthin was decomposed by treatment with acids, with formation of dextrose and *colocynthein*. G. Henke, on the other hand, stated that colocynthin was unaffected by dilute acids.

Concentrated sulphuric acid dissolves "colocynthin" to form a solution with orange-red colour, carbonisation occurring on heating. In concentrated hydrochloric acid "colocynthin" dissolves with coloration, and, on boiling, a dark green greasy substance is precipitated,

which, after drying over sulphuric acid, is only partly soluble in ether. The mother-liquor reduces Fehling's solution.

With sulphomolybdic acid, "colocynthin" is said by Johansson to give a cherry-red coloration; with sulphovanadic acid, a blood-red colour changing to blue at the edges; and a yellow coloration with alcohol and sulphuric acid (distinction from solanine and solanidine). Moistened with phenol and a drop of sulphuric acid, "colocynthin" gives a blood-red coloration changing to orange.

"*Colocynthein*" is not so soluble in water as "colocynthin," but is dissolved by ether and benzene, and is sparingly soluble in petroleum spirit. It may be extracted from acidified aqueous liquids by agitation with benzene, and the "colocynthin" can then be removed by ethyl acetate.

According to Power and Moore (*J. Chem. Soc.*, 1910, **97**, 99) "colocynthin" and "colocynthein" are not definite individuals. On extracting the pulp of *Citrullus colocynthis* (Schrader) with alcohol and subjecting the resulting extract to distillation with steam, a very small amount of a pale yellow essential oil was obtained. From the portion of the extract which was soluble in water the following substances were isolated:

(1) A new dihydric alcohol, $C_{22}H_{36}O_2(OH)_2$, (m. p. 285 to 290°) termed *citrullol*, which is apparently a lower homologue of ipuranol and yields a diacetyl derivative, m. p. 167°. (2) An amorphous, alkaloidal principle, which is a weak base and from which no crystalline derivative could be prepared; it possesses an extremely bitter taste and represents one of the purgative principles of the fruit. The aqueous liquid from which the above-mentioned compounds were isolated contained, furthermore, a quantity of inorganic salts, a little sugar and a very small amount of an amorphous glucosidal substance on which the purgative properties were found to depend.

The portion of the alcoholic extract which was insoluble in water consisted chiefly of resinous material, from which, however, a quantity of α -*clatrin* (m. p. 232; $[\alpha]_D - 68.9$) was isolated (compare *J. Chem. Soc.*, 1909, **95**, 1989). After the separation of the latter substance the resin was extracted with various solvents, which yielded a small amount of *hentriacontane*, $C_{31}H_{64}$, (m. p. 68°); a *phytosterol*, $C_{27}H_{46}O$ (m. p. 160 to 162°, optically inactive); a mixture of fatty acids and a further quantity of α -*claterin*, together

with a little of the above-described alkaloidal principle. None of the extracts from the resin was glucosidic. The ether and chloroform extracts possessed marked purgative properties, even after the removal of the active alkaloidal principle.

The seeds of the colocynth, which represented 75.5% of the entire peeled fruit, were found to contain traces of an alkaloidal principle, a small amount of an enzyme which hydrolyses β -glucosides, and a quantity of fatty oil corresponding with 12.72% of the weight of the seed. The constants of the fatty oil and of the total fatty acids obtained therefrom were determined, and from the oil a small amount of a phytosterol, $C_{20}H_{34}O$, was isolated, which melted at 158 to 160° and had $[\alpha]_D + 8.1^\circ$.

The amount of glucosidic substance in the fruit is extremely small. The purgative action is due to at least two principles, one of which is alkaloidal, although a very weak base and incapable, apparently, of forming any crystalline salts, whilst the other is represented by some non-basic principle or principles contained in both the ethereal and chloroform extracts of the resin. All attempts to obtain the last-mentioned active principle in a more definite form were, however, unsuccessful. No evidence could be obtained of the presence of β -elaterin, which constitutes the physiologically active constituent of *Ecballium elaterium*.

Colocynth pulp is obtained from at least three continents, but two principal varieties are recognised—Turkey colocynth and Spanish colocynth. Of these, the former is whiter in colour and contains a larger proportion of pulp. The fruit is collected when ripe, freed from rind and dried. Occasionally it is imported unpeeled.

The colocynth pulp of commerce is usually in the form of balls about 2 in. in diameter, often broken. Each fruit consists of a yellowish-white pith-like pulp, in which a large number of hard brownish-white seeds are contained, arranged in 6 vertical rows. The seeds, which are oil-bearing, make up 75% of the weight of the drug as imported.

The British Pharmacopœia (1914) defines colocynth pulp (*Colocynthis pulpa*) as the fruit and dry pulp of *Citrullus colocynthis*, freed from seeds, and the United States Pharmacopœia X (1925) as the dry pulp of the unripe full-grown fruit of *Citrus colocynthis* L. (Schrader). The B. P. describes it as white, spongy light fragments with thin-walled parenchymatous cells with no starch and few

sclerenchymatous cells. It has no odour, is very bitter and must contain not more than 2% of oil extractable by petroleum spirit (absence of large quantities of seeds), and must yield at least 9% of ash (seeds contain only 2-3% of ash). The use of petroleum spirit in place of ether for the extraction of the oil is due to Dowzard (*Pharm. J.*, 1903, **71**, 400), who pointed out that pure colocynth pulp yields about 3% of soluble matter when extracted with ether, and that this can scarcely be called a trace. The greater part of the ethereal extract, however, is "colocynthin," not oil, and Dowzard therefore recommended the use of petroleum spirit, in which "colocynthin" is insoluble. With this solvent, he never found more than 1.3% of oil in pure pulp, and he suggested that 2% might be fixed as the maximum amount permissible. The pharmacist is therefore required to separate the seeds, and this is done by sifting the opened fruits.

The U. S. Pharmacopœia requires not more than 5% of seed, 2% of epicarp, 2% of oil, or 6% of ash insoluble in acid. The pulp is used pharmaceutically in the form of an alcoholic extract of a mixture of it with aloes, scammony resin, powdered curd soap and condamon seed; hyoscyamus may also be added.

Chattaway and Moor (*Analyst*, 1903, **28**, 205) are in favour of reducing the standard of ash to 8%, and Umney (*Pharm. J.*, 1903, **71**, 879) says the ash in genuine samples may vary from 7.2 to 13.5%. Umney thinks that a microscopical examination (*cf.* British Pharmaceutical Codex) and a determination of the fixed oil are the best means for detecting adulteration.

David's modification (*Pharm. Z.*, 1928, **73**, 525) may be used for purely qualitative tests for colocynth, an extract of the sample being mixed into a paste with lime to form a small ball which is dried, powdered and extracted twice with methyl alcohol. A vivid carmine ring is produced if a layer of 2 c.c. of concentrated sulphuric acid is placed on the surface of a mixture of 1 drop of ferric chloride solution and a solution of the residue in 2 c.c. of glacial acetic acid.

The active principle of colocynth may be determined by washing the sample twice with 5 c.c. of ether, and decolorising a solution of the residue in 20 c.c. of warm absolute alcohol, with charcoal. The evaporated filtrate is extracted with chloroform, the solvent removed and 0.5 grm. of barium sulphate added to a solution of

the residue in 20 c.c. of water. The residue, after evaporation of the filtered solution, is dried at 60° till constant in weight. A sample of fruit yielded 25–30% of extract containing 6% of colocynthin, (Rózsa, *Chem. Zentr.*, 1928, ii, 701).

Bitters of *Cocculus Indicus*

The seeds of *Anamirta paniculata* or *Cocculus Indicus* contain several bitter principles, of which picrotoxin is the most characteristic.

Picrotoxin, according to Paternò and Ogliastro, confirmed by Schmidt and Löwenhardt, contains $C_{30}H_{34}O_{13}$. For the extraction of picrotoxin, Barth and Kretschy exhaust the cocculus berries with boiling alcohol or petroleum-spirit, evaporate the filtered solution, and extract the fatty residue with hot water. The fat (stearin) may be pressed out while hot. The aqueous extract is precipitated with neutral lead acetate, filtered, the filtrate treated with hydrogen sulphide, and the filtered liquid concentrated to a small bulk, when crystals of impure picrotoxin separate on cooling.

Barth and Kretschy believed the product thus obtained to be a mixture of picrotoxin, picrotin, and anamirtin, which compounds they obtained from it by repeated crystallisations from benzene or water;¹ but Paternò and Ogliastro regard all three substances as decomposition-products of true picrotoxin, and their conclusions are confirmed by the researches of Schmidt and Löwenhardt. These point out: (1) That true picrotoxin melts at 199 to 200°; (2) that picrotoxin does not lose weight when heated to 100°, whereas picrotoxinin loses its water of crystallisation (1 molecule); (3) that

¹ **Picrotin**, $C_{18}H_{18}O_7$ (Paternò and Ogliastro), is prepared by the action of hydrochloric acid on the ethereal solution of picrotoxin, picrotoxinin or picrotoxide, $C_{18}H_{18}O_8$, being simultaneously formed:



Picrotin is also formed when a chloroform solution of picrotoxin is allowed to stand in the cold, or by the action of bromine water on a hot aqueous solution of picrotoxin, followed by filtration and evaporation, whilst by boiling an aqueous or benzene solution of picrotoxin that substance is split up into picrotin and picrotoxinin. Picrotin crystallises with varying quantities of water. When heated it darkens at 245°, and melts at 250 to 251°. It has a very bitter taste, but is not poisonous. Picrotin reduces ammonio-silver nitrate and Fehling's solution. Its solution in benzene is unaffected by boiling or by hydrochloric acid, but it is readily decomposed by alkalis. With strong sulphuric acid, picrotin develops a pale yellow colour, which changes to orange on heating. It forms numerous derivatives.

Picrotoxinin, $C_{18}H_{18}O_6$, H_2O , is also formed by the action of hydrochloric acid on picrotoxin or by the reduction of bromopicrotoxin ($C_{18}H_{18}O_6Br$, colourless prisms m. p. 260°). It crystallises in rhombic plates, which become anhydrous at 100°, and melt at 200°. Picrotoxinin is bitter and very poisonous. It dissolves readily in hot water, alcohol, ether, chloroform, and benzene. It is reduced by Fehling's solution and ammonio-silver nitrate, and gives colour reactions similar to those yielded by picrotoxin.

Picrotoxide is an isomer of picrotoxinin produced from it in ethereal solution by the action of hydrochloric acid, m. p. 309°.

See also Meyer and Bruger, *Angelico, Gazzetta*, 1906, 36, [ii] 645; *Gazzetta*, 1909, 39, [ii], 206; *Gazzetta*, 1910, 40, [i] 391; *Ann. R. Accad. Lincei*, 1910, [v], 19, i, 473; and Horrmann and Prillwitz (*Arch. Pharm.*, 1920, 258, 200).

picrotoxin, when treated with a large quantity of benzene in the cold, expands, whereas picrotin and picrotoxinin do not, and (4) that picrotoxinin, so treated with benzene, undergoes no change in its composition, which would scarcely be the case were it a *mixture* of the two, since picrotoxinin is soluble to the extent of 3.5 in 1,000 of benzene, whilst the solubility of picrotin is only 0.2 in 1,000. The name picrotoxin is therefore applied to the original substance, and picrotoxinin to Barth and Kretschy's product.

The appearance and crystalline form of picrotoxin vary with the conditions of separation. Thus, if a fairly concentrated solution be evaporated slowly, the picrotoxin separates in well-defined yellow prisms; but if the solution be evaporated rapidly and cooled quickly, characteristic feathery forms are deposited. When the solution is dilute, picrotoxin separates in long radiating needles. According to the United States Pharmacopœia, picrotoxin occurs as colourless, shining, prismatic crystals, or as a micro-crystalline powder.

Picrotoxin is odourless, permanent in the air, and intensely bitter. It melts at 200° to a yellow liquid, at a higher temperature evolves acid fumes having an odour like that of caramel, and ultimately chars. If it is sublimed at $215-225^{\circ}$ it is obtained in droplets, and not crystals, which are soluble in nitric acid without production of colour, and which, when warmed gently with a 5% solution of ferric chloride almost to the boiling point, yield characteristic colourless pentagonal crystals distinct from those given by antipyrine.

Picrotoxin dissolves very sparingly (1:200) in cold water, more readily in hot (1:25), and is very soluble in boiling alcohol. It is also soluble in glacial acetic acid, amyl alcohol, and benzene, but is only sparingly soluble in ether or chloroform. It dissolves in ammonia and in acidified water.

Picrotoxin may be extracted from acidified aqueous liquids by agitation with chloroform, ether, or amyl alcohol, the first solvent being preferable. Benzene is stated not to extract it.

The alcoholic solution of picrotoxin is neutral in reaction and lævorotatory, but the optical activity is very variously stated.

Picrotoxin is neutral to litmus, but appears to possess feeble acid properties, since it is not extracted from aqueous liquids by agitation with immiscible solvents in presence of alkalies. It forms crystallisable compounds with certain of the alkaloids.

Picrotoxin is not a glucoside, but it reduces Fehling's solution gradually in the cold and more rapidly on heating, the value of K being about 20. It also reduces silver from the ammonio-nitrate and is readily decomposed by boiling alkali hydroxides.

When a mixture of picrotoxin with an equal weight of sodium hydroxide is moistened with a drop of water, a green coloration gradually changing to reddish-brown, is produced. It dissolves in cold concentrated sulphuric acid, with a bright yellow colour, darkening to orange-red on warming and changing very gradually to reddish-brown. The liquid exhibits a brownish fluorescence. The addition of solid potassium dichromate to the solution in cold acid causes a change in colour to green.

Picrotoxin gives no coloration with nitric acid of sp. gr. 1.2, but it dissolves in acid of sp. gr. 1.4, and the liquid leaves on evaporation a reddish-yellow residue which becomes bright red when moistened with alkali hydroxide.

If picrotoxin is treated with strong sulphuric acid, and a minute crystal of nitre added, the mixture gives a coloration varying from red to violet on addition of excess of strong alkali hydroxide.

A mixture of picrotoxin with cane-sugar becomes red on treatment with strong sulphuric acid.

Aqueous solutions of picrotoxin are unaffected by auric, platinic, or mercuric chloride, Mayer's reagent, tannin, potassium ferrocyanide and ferricyanide, and most other of the general reagents for alkaloids.

Picrotoxin is not precipitated by either neutral or basic lead acetate. R. Palm has pointed out (*J. Pharm. Chim.*, 1888, [v], 17, 19) that if an aqueous or alcoholic solution of picrotoxin is shaken vigorously with recently-prepared and well-washed lead hydroxide (prepared by precipitating a solution of lead acetate with ammonia), the bitter principle is completely removed from solution. If the precipitate is separated, dried gently, and treated with cold concentrated sulphuric acid, a bright yellow coloration will be produced, changing to reddish-violet on warming.

Picrotoxin has an intensely bitter taste, and is very poisonous. Fish appear to be specially susceptible to its action. When introduced into a very dilute solution of picrotoxin, the fish swim with uncertainty, lose their balance, and ultimately rise to the surface, lying on their sides and frequently opening their mouths and gill-

covers. These symptoms, however, are by no means peculiar to poisoning by picrotoxin.

A. Wynter Blyth considers frogs to be more sensitive than fish to the effects of picrotoxin. The frogs "become first uneasy and restless, and then somewhat somnolent; but after a short time tetanic convulsions set in, similar to those observed in poisoning by strychnine, but with picrotoxin an extraordinary and highly characteristic swelling of the abdomen occurs." The physiological properties of picrotoxin are destroyed by the addition of blood or of urine.

In toxicological inquiries, picrotoxin will be extracted when a concentrated alcoholic extract of the material is acidified and shaken with chloroform or ether. From the residue left on evaporating the solvent, the picrotoxin may be dissolved out by hot water, and crystallised from the solution on concentration. Or the aqueous solution may be treated with neutral lead acetate, excess being avoided and the filtered liquid shaken with freshly prepared lead hydroxide. The precipitate may be decomposed with dilute sulphuric acid, and the picrotoxin extracted with ether, or reagents may be applied to the precipitate itself.

Minovici (*Ann. Pharm.*, 1901, 7, 1) recommends anisaldehyde as a very delicate and characteristic reagent for picrotoxin, particularly when present in the starchy mass of the fruit. A drop of sulphuric acid is added to the sample and, after 2 minutes, when the saffron-yellow colour has become distinct, 1 drop of a 20% solution of anisaldehyde in absolute alcohol is introduced. A dark blue-violet ring is produced which soon attains a stable pure blue. The test can also be applied to dilute solutions (1 in 4,000) in chloroform, alcohol or water, but the liquids should previously be warmed. Many glucosides and alkaloids give colour reactions with the reagent, but the red-blue given by veratrin cannot be mistaken for the permanent blue given by picrotoxin, and the colour given by the other substances which react with anisaldehyde is in nearly every case a shade of red.

Cocculus Indicus, or *Anamirta paniculata*, the berries of which are the source of picrotoxin, is a small climbing shrub growing in India and the Malay Archipelago. In India the berries are employed as a drug, and picrotoxin itself is official in the United States Pharmacopœia, having replaced the powdered cocculus of earlier editions. Cocculus also forms an ingredient of an ointment having a very

limited use. The berries have been used by fish-poachers for poisoning fish, and a preparation known as "Barlow's poisoned wheat" is stated to owe its active properties to the presence of cocculus.

From 2 to 3 grains of picrotoxin, or about 100 grains of cocculus, cause spasms and other symptoms suggestive of strychnine. Strong contraction of the uterus has been observed. Chloroform acts in antagonism to picrotoxin, and prevents the spasms caused by moderate doses.

In a number of recorded cases cocculus berries have proved fatal to human beings. In 1829, several men, of whom one died, were poisoned by drinking rum containing a preparation of cocculus. In another case, a boy aged 12 was persuaded to swallow a powder containing cocculus used for poisoning fish. He suffered intense pain throughout the whole length of the alimentary canal, followed by fever and delirium, and died on the nineteenth day. The *post-mortem* symptoms were those of peritonitis. (See also Government of Madras, official report of Chemical Examiner, 1925.)

Cocculus Indicus berries contain from 1 to 2% of picrotoxin, together with cocculin and anamirtin (?).¹ The husks contain a non-poisonous alkaloid called menispermine,² but no picrotoxin.

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HOPS

The hops of commerce are the ripened and artificially dried female flowers of *Humulus lupulus*, a dioecious plant belonging to the order

¹ Anamirtin is regarded by Paternò and Ogliaro (see p. 110) as a product of the decomposition of picrotoxin, and not as a natural constituent of the cocculus berries. It remains in the mother-liquor when picrotoxin is crystallised from water. Anamirtin, $C_{15}H_{24}O_{14}$, forms short needles. When heated to 280° it chars without melting. It is free from bitter taste, and is not poisonous. It dissolves in water, but is only sparingly soluble in chloroform or benzene. Anamirtin does not reduce Fehling's solution or ammonio-nitrate of silver.

² Cocculin, $C_{10}H_{18}O_{10}$, is regarded by its discoverer, Löwenhardt, as probably identical with the anamirtin of Barth and Kretschy. It crystallises in small white needles, sparingly soluble in hot water, alcohol, or ether, and insoluble in cold water or ether, but slightly soluble in cold alcohol. It is not bitter, and it does not give the colour reactions of picrotoxin.

³ Menispermine, $C_{15}H_{24}O_2N_2$, forms white, four-sided prisms, m. p. 120°. It is insoluble in water, but dissolves in warm alcohol or ether. Menispermine has no bitter taste, and is not poisonous. It is a well-defined base with alkaline reaction.

Paramenispermine is separated from menispermine by treating the mixed bases with ether, in which the former is insoluble. It forms quadrilateral prisms or radiating crystalline masses. It melts at 250° and at a higher temperature sublimes unchanged. The crystals are insoluble in water, and nearly so in ether, but dissolve in absolute alcohol.

Hypopicrotoxic acid, a brown mass, m. p. 100°, is also present.

Cannabaceæ. The female flowers, which are very small and very simple in character, consisting of a cup-shaped corolla with a round ovary containing one seed, grow together in structures somewhat resembling fir-cones and technically termed "strobiles." On dissection, each strobile or "hop" will be found to consist of a number of leaf-like bracts and bracteoles, termed by hop-growers "petals" or "scales," arranged on a central axis or "strig." The bracts are arranged on the strig in 4 rows and in reality are pairs of stipules of leaves whose green leaves usually remain undeveloped. Just above each pair of these bracts, short branches grow out from the central axis, and upon each branch are produced 4 female flowers, each surrounded by a bracteole. The ripened hop thus consists of a strig, n bracts, $2n$ bracteoles and $2n$ fruits or abortive ovaries, n being any number from 8 to 50, 15 being perhaps the average for English hops. The fertilised and ripened fruit (an achene containing a single seed) is sometimes termed in the trade a "seed." Continental hops are almost seedless, owing to the absence of male plants, and even English Goldings and American varieties, which do not develop well unless a fair proportion of the flowers are allowed to become fertilised, contain a large number of abortive ovaries, which are small, shrivelled, inconspicuous structures.

The bracts and bracteoles are of approximately the same size, but differ in shape and texture. The bracts are the more symmetrical and are rather coarser in texture and more thickly veined than the bracteoles, which are usually slightly longer than the corresponding stipular bract. Toward its base, on one side, the bracteole is folded on itself and encloses the fruit if one has developed. The bracteoles are not only longer but narrower at the tip than the bracts, the upper parts of which are broader as a whole, though suddenly becoming acuminate at the actual tip. The bracteoles are also of a brighter yellow colour than the bracts when the hop is ripe.

On examining the dissected parts of a hop-cone with a lens, a large number of pollen-like grains will be observed attached to the outer surface of the bracteoles, especially near their base. - The pericarp, enclosing the seed, is also thickly covered with them, and, in the better class of hops, they will be found on the stipular bracts as well. Botanically these structures are regarded as glandular trichomes or hairs, and they are spoken of by growers and brewers as "hop-meal," "hop-flour," "pollen," or "condition." The Pharmacopœias (British and United States) called the hop-meal "lupulin," and the

expression "lupulin glands" is very general among chemists. In England, the glands make their appearance toward the end of July or beginning of August as small cup-shaped structures, which become more and more filled with oily, resinous contents as the season advances. The oily material is, for a time, transparent, and of a clear golden yellow colour resembling amber varnish, but when the glands are dead ripe the contents become turbid and opaque and are not unlike flowers of sulphur. In commercial samples they always appear transparent, as hops are usually picked unripe and, even if allowed to ripen, the subsequent drying process once more restores to the glands their transparent appearance. The glands darken in colour and suffer other visible changes on storage or when dried carelessly, but a consideration of these changes is best deferred until, towards the end of this section (see Physical Examination of Hops), the physical characteristics of a good hop are considered.

For a fuller account of the morphology of the hop, the reader is referred to an article by Percival in a Supplement to the *Brewers' Journal*, 1902, 38, March. The varieties of English hops are also described exhaustively by Percival (*J. Roy. Agric. Soc.*, Vol. 62). Choice hops are also produced on the Continent, particularly in the Saaz and Spalt districts, and also in the United States and British Columbia. The latter, in fact, tend to rival the best East Kent hops.

Constituents of the Hop

The principal constituent of the hop is *lupulin*. It does not occur in current editions of the British or United States Pharmacopœias, but was previously defined in an earlier edition of the former as glands obtained from the strobiles of *Humulus lupulus*. It should contain not more than 40% of matter insoluble in ether, and yield not more than 12% of ash when incinerated. The latter required lupulin to contain at least 60% of matter soluble in ether and placed the limit of ash at 10%. The hop (*Humulus*) of the same edition is defined as the carefully dried strobiles of *Humulus lupulus* L., bearing their glandular trichomes, and without the presence or admixture of more than 2% of stems, leaves or other foreign matter.

The British Pharmaceutical Codex (1907) describes lupulin at length, and, in particular, states that the glands may be separated by shaking and beating hops, and that they readily burst and discharge

their granular oleoresinous contents on the application of slight pressure. It also adds the warning that the commercial article may be kiln sweepings, and that a dark colour and disagreeable odour are an indication of age. It is further stated that some samples may contain as much as 25% of ash and more than 40% of matters insoluble in ether.

The value of hops to the brewer depends, almost entirely, on the contents of the lupulin glands, since they contain not only the preservative resins and bitter acids, but also the essential oil on which the aroma of the hop, and indirectly the flavour of hopped worts, depend.

The percentage of lupulin in good hops approximates to 20%, though it is rarely possible to separate so much mechanically. On extracting lupulin with ether, from 60 to 80% may dissolve. The ethereal extract is mainly made up of the resins and bitter substances, the insoluble portion of cellular tissue and sand. Barth ("The Hop and Its Constituents," ed. Chapman, page 81) gives the following analysis of the matters insoluble in ether from a commercial sample.

Ash, insoluble in hydrochloric acid,	15.3
Ash, soluble in hydrochloric acid,	2.7
Proteins ($N \times 6.25$)	4.8
Pentosans (by Tollens' method),	2.3
Crude fibre, tannin and loss (by difference),	10.9
	<hr/>
	36.0%

The constituents not determined, or given only as a difference number, include tannin (2.5-6.0%) and phlobaphen. The latter is supposed to form an insoluble compound with the proteins of the wort during the boiling process. The hot-water-soluble proteins amount to 0.5-1.0%. In addition to carbohydrates and fibrous matter (6.11%), moisture, and traces of diastase, there is also evidence of the presence of morphine and choline. Certain American wild hops certainly do contain morphine and another substance of alkaloidal character to the extent of 0.15%, but not more than infinitesimal traces of these substances have been found in cultivated hops (Williamson, *Chem. Zeit.*, 1886, 10, 20, 38 and 147, and Ladenburg, *Ber.*, 1886, 19, 783). Lecithin also is, no doubt, present in

hops, though probably in small quantity in ripe hops, and would in part pass into the ethereal extract. It is probable that the choline obtained from hops by Griess and Harrow (*Ber.*, 1885, 18, 717) was not present as such in the hops, but resulted from the splitting up of lecithin.

Barth, in his analysis of lupulin, groups together γ -resin, essential oil and fatty acids. Chapman (*J. Chem. Soc.*, 1895, 67, 55), who submitted some hundredweights of hops of various origin to steam-distillation, obtained only about 0.2% of essential oil, equivalent to about 1% on the lupulin. The "seeds" of hops contain upwards of 25% of a fatty oil, and kiln-sweepings, which are said to be sometimes sold as lupulin, may therefore contain fatty oil derived from ruptured seeds. The structure of the hop cones must protect the seeds of pocketed hops in ordinary circumstances, for crushed seeds are rarely found, but Briant (*J. Inst. Brew.*, 1910, 16, 5) has shown that in exceptional cases the seeds may be ruptured. Barth's lupulin, however, was specially selected for use in an investigation on hop resins, and it is improbable that it contained any considerable amount of oil derived from seeds. The insoluble ash of lupulin varied from 10 to 20% in a number of samples examined by Barth (*loc. cit.*), whilst at least one sample has been described as containing only 4% of total ash.

Other than in the brewing industry the uses of the hop are few and unimportant. Its medicinal value is defined by its description in the B. P. Codex (1923) as that of an aromatic bitter and mild sedative, the properties of which reside in the lupulin. Hop pillows are supposed to induce sleep, and hop-powder has been mixed with sulphur, asafoetida and sago flour, and used as a denaturant for tobacco. Unused hop fibres have also been used in the textile industry, and several outlets for spent hops exist. Thus, they are incorporated in cattle-foods, though Davies (*J. Agric. Sci.*, 1927, 17, 380) states that, in spite of their favourable analytical numbers, they are not readily digested. In the dried state they are used in filter-beds to adsorb molasses.

The principal functions of hops in brewing are:—

- (1) The preservation of the beer.
- (2) The production of the bitter hop aroma.
- (3) The coagulation of the protein and other substances thrown out of solution on boiling.

They also contribute towards head-formation and retention by virtue of the lowering in surface tension produced by the constituent humulon (see below), this substance and its soft resin transformation products being 4 times as effective as the other constituent lupulon (Windisch, Kolbach, and Banholzer, *Woch. Brau.*, 1926, **43**, 207 *et seq.*). Since the lupulin contains the volatile oil and the bitter substances on which the aroma of the hop chiefly depends, there is a tendency to neglect the other constituents of the hop. Thus, adverse flavours may be produced by tannins in the presence of alkaline waters, by quercetin glucosides which may produce acidity, or by organic sulphur compounds formed as a result of the use of an ammonium sulphate fertiliser.

The oil of the hop, which is devoid of antiseptic properties, occurs to the extent of 0.2–0.8% and is soluble in alcohol,¹ ether or in 600 parts of water. Chapman (*J. Chem. Soc.*, 1895, **67**, 54, 780; 1903, **83**, 505) fractionated the oil from several hundredweights of hops of different origin and found that each sample yielded 40 to 50% of an unsaturated aliphatic hydrocarbon, boiling at 166 to 168°, two small intermediate fractions, and about 40% of a sesquiterpene boiling at 263 to 266°. The sesquiterpene, to which Chapman gave the name of *humulene*, $C_{15}H_{24}$, possesses little odour and must be regarded as a diluent of the oil. It has not been detected in any other natural source, although Deussen (*J. pr. Chem.*, 1911, **83**, 483) has claimed that it is identical with the inactive or α -caryophyllene isolated by the fractional distillation of clove oil. Chapman, however, (*J. Chem. Soc.*, 1928, 785, 1303) admits that these substances are similar, but quite definitely rejects the suggestion that they are identical. The intermediate fractions consisted mainly of inactive linalool and linalyl isononoate, both of course fragrant substances, whilst the unsaturated aliphatic hydrocarbon, constituting the first fraction, was subsequently shown to have the formula $C_{10}H_{16}$ and to be identical with *myrcene*,¹ first obtained by Power and Kleber from bay oil. Myrcene has a penetrating odour which is not unpleasant, but which is quite distinct from that of the unfractionated hop oil. Essential oil of hops also contains a small amount of a diterpene, probably some ester of geraniol, and probably other esters.

¹ One part of fresh hop oil shaken with 9 parts of 90 % alcohol produces a haze on account of the large proportion of myrcene. Old oil, however, which has become oxidised, remains bright.

Recent work (*loc. cit.*) has also shown that the higher-boiling fractions of the oil (b. p. 87–200° at 4 mm.) could be divided into seven sub-fractions. The most important of the new substances obtained were a ketone *luparone*, $C_{13}H_{22}O$, (b. p. 74–76° at 3 mm.), and an unsaturated alcohol *luparenol*, $C_{15}H_{24}O$, (b. p. 125 to 128° at 3 mm.), whilst the residue yielded *luparol*, $C_{16}H_{26}O_2$, (b. p. 122–124° at 2 mm.). Luparol gives a deep red colour with ferric chloride, and decomposes into *iso*-valeric acid, a constituent of the original oil. These substances occur only in small quantities, but their combined effect is responsible for the aroma of the oil.

Brewers add hops not only to their boiling wort, but also to some of their finished beers when in the trade casks, and use for this purpose always the finest hops. As a result of this "dry-hopping," a very small quantity of essential oil does, no doubt, find its way unchanged into the beer and contributes to the flavour. But what determines the difference in flavour of, say, Californian, Bavarian and Kentish hops is unknown. Even an inexperienced person can at once recognise that their odours are very dissimilar, yet Chapman isolated the same constituents in not very different proportions from oil derived from each of these. Rabak (*J. Agric. Res.*, 1914, 2, 115), on the other hand, found that to a certain extent the constants of hop oils were characteristic of the locality in which the hops were grown, and that this was most true of the ester value. Lüers (*Brewers' J.*, 1928, 64, 138) has carried out experiments to test the effect on the taste of the beer of the particular type of hop used. The panel of judges decided that, as far as hop flavour itself was concerned, it was impossible to identify a hop from the finished beer, though the bitter flavour is more characteristic of the origin of the hop. Even so, this power of discrimination is no doubt confined to the expert.

Since the oil is steam-volatile, and disappears during the boiling process particularly where open coppers are used, products are marketed which are added to the finished beer (see p. 148). The flavour and bitterness produced in the wort by the hop are also removed by other agencies, (Wiegmann, *Pet. Jour. du Brass.*, 1924, 32, 919, 1082). Phosphates, dextrin and maltose all tend to reduce bitterness, and whilst the two latter are removed during fermentation, it is found that worts made with soft waters require more hops than if hard waters are used, since the carbonates in the latter

combine with the phosphates. These influences account for almost 45% of the bitter substances, whilst the spent hops and coagulation during the cooling account for the removal of about the same percentage. Hence it is customary also to add a few fresh, choice hops near the end of, or after the boiling process. The rôle of hop oil in brewing is fully discussed by Chapman (*J. Inst. Brewing*, 1929, 36, 247).

Bitter Substances.—The principal early work on the bitter substances of the hop is due to Hayduck (*Woch. Brau.*, 1888, 937) who showed that the ethereal extract of hops, when freed from a wax previously described by Lerner, contained at least three resins and two crystallisable substances. Two of these resins and both the crystallisable substances were soluble in petroleum spirit, whilst the remaining hard, tasteless resin was not. Hayduck extracted hops with ether, evaporated the ethereal extract, and treated the residue with alcohol. A wax (Lerner's "myricin") remained undissolved. From the alcoholic solution, one of the resins, called by Hayduck the α -resin, was precipitated as a lead compound by addition of an alcoholic solution of lead acetate. By decomposition of its lead compound, the α -resin was obtained as a soft, bitter resin, soluble in petroleum spirit and sparingly soluble in water and in dilute alcohol.

In the filtrate from the lead compound of the α -resin, the remaining two resins were found. These were separated by taking advantage of the fact that one was soluble, the other insoluble, in petroleum spirit. The resin soluble in petroleum spirit but not precipitated by lead acetate was a soft, bitter resin, sparingly soluble in water or dilute alcohol, and was called by Hayduck the β -resin, whilst the hard, tasteless resin, insoluble in petroleum spirit, was called the γ -resin.

Hayduck made the further observation that, under certain conditions, microscopical crystals separated from the α - and β -resins. The crystals from the α -resin differed notably in appearance from those yielded by the β -resin, but both were intensely bitter substances slightly acid in character. Hayduck's crude α - and β -resins must, therefore, each be regarded as a mixture of at least one resin and one crystallisable substance, unless it be assumed that the crystallisable substances are derived in some manner from the resins after the separation of the latter by Hayduck's method. This assumption is scarcely tenable in view of the marked tendency of

each of the crystallisable substances to become transformed into a resinous mass, indistinguishable from the resin from which the crystals were first separated. Hayduck himself expressed the view that the resins proper were simple polymers of the crystallisable acids, and that each of his so-called α - and β -resins was a mixture of the true resin with unpolymerised bitter acid. H. Bungener (*Bull. Soc. Chim.*, 1886, **45**, 487) had at an earlier date expressed the view that the resin of hops—then supposed to be a single substance—was an oxidation product of his crystalline acid (the β -acid), and other authors have suggested that the resins may result from the polymerisation of some oxidation product of the crystalline acids.

Lupulin.—In 1920 the Research Committee of the Institute of Brewing inaugurated investigations of the constituents of the hop of value in brewing. The greater part of this has been carried out by Pyman, Walker and co-workers and is embodied in a series of Reports (*J. Inst. Brewing*, 1922–29). It was realised at the outset that, since the preservative property of the hop is associated with the resinous constituent of the lupulin, an exact knowledge of the chemical constituents and properties of the lupulin was desirable. Work was commenced on these lines and several methods evolved for the evaluation of hops (see p. 126). Work carried out in Germany by Windisch and his co-workers, on parallel lines, has led to substantially the same results.

Humulon and Lupulon.—Lupulin has long been known to contain (a) a hard resin of little or no preservative power, soluble in ether and insoluble in petroleum spirit; (b) a soft resin, soluble in petroleum spirit, and separable into an α - and β -resin which contain the crystalline substances humulon and lupulon, respectively. These two substances, particularly lupulon, are bitter and have antiseptic properties. Chapman (*J. Chem. Soc.*, 1914, **105**, 1895) also detected nitrogenous compounds (histidine, adenine, hypoxanthine and betaine) in the soft resin, whilst among the non-nitrogenous constituents Power, Tutin and Rogerson (*id.*, 1913, **103**, 1267) detected dextrose, phytosterol and the two phenolic substances, humulol ($C_{17}H_{18}O_4$) and xanthohumulol ($C_{13}H_{14}O_3$), both of which are probably products of hydrolysis. Many attempts have been made to obtain humulon in the pure state, that of Wöllmer (*Ber.*, 1916, **49**, 780), who extracted the resin with methyl alcohol and precipitated it several times with lead acetate followed by extraction with dilute

sulphuric acid, being the foundation of the present methods. As a final stage a compound of *o*-phenylenediamine and humulon was formed, purified by recrystallisation, and decomposed by hydrochloric acid. Barth (*Z. ges. Brauw.*, 1900, **23**, 509) recommends Bungener's method (*loc. cit.*, p. 122) for the extraction of lupulon, in which the petroleum spirit extract is rapidly evaporated *in vacuo* (to arrest resinification) and recrystallised from petroleum spirit and finally from methyl alcohol. The early work is described and discussed by Walker and others (*J. Inst. Brewing*, 1922, **28**, 929; 1923, **29**, 373).

It was realised later (*id.*, 1924, **30**, 570, 712) that extraction of the soft resin with petroleum spirit is slow and incomplete, on account of the coating of hard resin surrounding the lupulin, and that it was preferable to use this solvent (or *n*-hexane) to extract a dilute methyl alcohol solution of the resins. By these means it was possible to eliminate the adventitious matter (about 2%) associated with the weakly acidic α - and β -resins, and to prepare crystalline samples of humulon and lupulon.

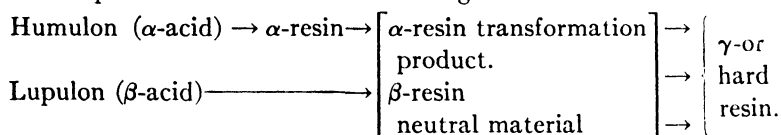
Humulon (humic acid, α -bitter acid), $C_{21}H_{30}O_5$, m. p. 65–66.5°, was shown by Wöllmer (*loc. cit.* and *Ber.*, 1916, **49**, 780) and by Wieland and others (*id.*, 1925, **58**, 102, 2012; 1926, **59**, 2352) to be an optically active unsaturated compound, acidic by virtue of an enolic group, and forming humic acid ($C_{15}H_{22}O_4$) and hexenoic acid ($C_6H_{10}O_2$) by the action of alkali; it is reduced to *iso*-pentane and humulohydroquinone, $C_{16}H_{24}O_5$.

This work led to the determination of the chemical constitution of *lupulon* (lupulic acid, β -bitter acid), $C_{26}H_{38}O_4$, and the work of Wöllmer (*Ber.*, 1925, **58**, 672), though not final, enabled a structure to be assigned to it. Lupulon (m. p. 95°) is a weak, monobasic, unsaturated acid, optically inactive, insoluble in cold water, but readily soluble in organic solvents. It resists alkaline hydrolysis, but acids decompose it into *iso*-amylene, an isomeric substance, and other compounds. It differs from humulon in that a hydroxyl group is replaced by an amylene residue.

Once the nature of these important substances was settled it was necessary to determine their individual influences and changes during the brewing process. It appears that the α - and β -acids tend to form the corresponding resins, a change which is quick and accelerated by oxygen and carbon dioxide in the latter, but not in the

former case (Windisch, Kolbach and others, *Woch. Brau.*, 1924, **41**, 175 *et seq.*; 1926, **43**, 349 *et seq.*). The increase in size of the colloidal particles of humulon on the addition of water to alcoholic solutions also increases its foaming capacity and its susceptibility to oxidation at first, but both these properties fall off after complete precipitation. The same authors showed (*ibid.*, 1927, **44**, 285 *et seq.*, 453) that during the boiling of the wort, resinification occurs, with the formation of hard resins at a rate which indicates a uni-molecular reaction, and that α -resins may also be formed.

The relation of the constituents which exist in or may be produced from lupulin are shown in the following scheme:—



The square brackets indicate the β -fraction. Kolbach (*Woch. Brau.*, 1925, **42**, 21) summarises the solubilities as follows:—

α -Products	β -Products	Solubility			Notes
		Alcohol	Ether	Petroleum spirit	
α -Bitter acid.....	β -Bitter acid.....	x	x	x	ppd. by Pb Ac.
α -Soft resin.....	β -Soft resin.....	x	x	x
α_1 -Hard resin.....	β_1 -Hard resin.....	x	x	Alc. sol- uble, not bitter.
α_2 -Hard resin.....	β_2 -Hard resin.....	x

He believes that the original lupulin consists of humulon and lupulon, and that during the drying, curing and boiling processes etc. the soft and hard resins increase at their expense. The course of this change is shown by the two first vertical columns.

It now became of importance to determine the relative antiseptic powers of these constituents in order to find a means of evaluation of the hop as a whole. Walker (*J. Inst. Brewing*, 1925, **31**, 562) showed that the α -fraction (impure humulon) of a soft resin had a preservative power about 4 times that of the β -fraction, the most active portion of which was lupulon. As lupulon occurs in smaller quantities than humulon, the relative activity of the β -fraction is low, in spite of the fact that the latter is nearly twice as active as the former.

Walker fractionated the resins according to their solubilities in solutions of various alkalies and showed the activities of the fractions to vary from $\frac{1}{4}$ to $\frac{1}{6}$ of that of lupulon. The neutral material was almost inactive.

These results confirmed the conclusions of Kolbach (*loc. cit.*) and also those of Brown and Clubb (*J. Inst. Brewing*, 1913, **19**, 261) and of Ford and Tait (*id.*, 1924, **30**, 426; 1926, **32**, 19). The latter authors had, in fact, devised an important method based on Siller's investigations (*Z. Unters. Nahr. Genussm.*, 1909, **18**, 241) in which the antiseptic power was given in terms of the percentages of α - and β -resins by the expression $(\alpha + \beta/5)$. This was subsequently changed by them to $(\alpha + \beta/4.3)$, and by Walker to $(\alpha + \beta/3)$. These latter expressions, which agree for new hops better than for old, appear to depend on the working procedure, as when this is standardised according to the method described on p. 128, concordant results are obtained. The work of Windisch, Kolbach and others (*Woch. Brau.*, 1924, **41**, 227; 1925, **42**, 281 *et seq.*; 1927, **44**, 285 *et seq.*) also showed that the resin formation, which is slow at the normal pH of wort, is accelerated in alkaline solution. The hourly decomposition of humulon by boiling water varies from 18% at pH 6.5 to 45% at pH 8.5. In the case of lupulon the variation is less, whilst the antiseptic power of the complete hop itself is a maximum at pH 5.5–6.8 (Fernbach and Stoleru, *Ann. Brass. et Dist.*, 1924, **23**, 1), and falls off rapidly for more alkaline solutions. Hops contain buffer substances which, with those of the medium, ensure a pH within this range under actual working conditions. In the formula of Brown and Tait $(\alpha + \beta/n)$, n will therefore vary widely with the pH.

On the basis of the above knowledge several reliable methods for the evaluation of the hop have been described.

Before proceeding to these, however, it should be stated that hops intended for use in brewing, are dried on kilns (oasts) over coal fires, on which, in Great Britain at any rate, it is customary to throw some sulphur. The reasons for the use of sulphur will be discussed when we come to consider its detection. In Great Britain, drying generally succeeds picking as fast as the capacity, often inadequate, of the oasts permits. With some Continental hops, on the other hand, a very considerable time elapses between picking and curing. The dried hops are finally pressed into bales (pockets) and are then ready to be marketed.

Commercial Analysis and Valuation of Hops

Sampling is a matter of some difficulty with a heterogeneous substance such as hops. Some years ago, Lintner (*Chem. Ztg.*, 1908, 32, 1068) and Siller (*Z. Unters. Nahr. Genussm.*, 1909, 18, 241) stated that, unless hops were finely comminuted, petroleum spirit failed to extract the soft resins completely, and it has become customary on the Continent to pass hops through a mincing machine before proceeding with this determination (see Neumann, *Woch. Brau.*, 1910, 27, 281). With Continental hops, which are substantially free from seeds, there is no objection to this, but with seeded British or American hops such treatment ruptures the seeds, and liberates the fatty oil contained in them and is not advisable for use with gravimetric methods. Otherwise a representative 30 grm. portion is removed from the broken package, and passed through an ordinary household mincer. The first 5 grm. are rejected as "rinsings," and the remainder is well mixed and is used for all the determinations, except those of moisture and arsenic, for which unground samples are required.

Antiseptic Value.—It is apparent that the most valuable information as to the quality of a hop will be derived from those methods which enable its preservative value to be measured most satisfactorily. The preservative value, however, is not the sole criterion of quality in a hop, since its properties, as far as the brewer is concerned, depend on the combined effect produced by all its constituent substances. Until recently, the determination of the hard and soft resins has provided the nearest approach to these requirements, but it will be apparent from the work described above that the resin-content is not a measure of preservative value, nor does it even provide true relative figures.

A biological method in which antiseptic value is measured as such, is obviously a nearer approach to the conditions of practice. Several have been proposed, and the two most important will be described.

Chapman's Biological Method (*J. Inst. Brewing*, 1925, 31, 13): This is based on the work of A. Brown and his co-workers (*id.*, 1910, 16, 64) in which the inhibition of the growth of *Bacterium* "X" (an organism isolated from acid beer and similar to *Van Laer's Saccharobacillus pastorianus*)¹ by varying quantities of an aqueous

¹ Since named *Bacterium C.*

hop extract, in a malt-extract medium is measured. Chapman's final method is an improvement, in that it is simple and rapid, the organism used is more sensitive to the inhibiting effects of hop extract, and the method provides a sharper end-point.

The broken hops (5 grm.) are infused with 950 c.c. of hot distilled water in a wide-necked graduated flask immersed in boiling water for $1\frac{1}{4}$ hrs., and the liquid cooled, filtered through glass wool and made up to 1 litre. Alternatively, a decoction may be prepared by Walker's method (p. 128). Measured volumes of the filtered extract are pipetted into a series of test-tubes and sufficient melted, sterile nutrient agar added to produce a volume of 10 c.c. in each case. The contents of the tubes are mixed, plugged, sterilised in steam for 10 minutes, cooled to 40° , and 3 drops added of a vigorous culture of an aerobic, spore-forming bacillus of medium length isolated from raw sugar. The *pH* value should be about 6.2 (see Fernbach and Stoleru, *loc. cit.*, p. 125). Each mixture is then poured on to a sterile Petri dish (3 in. in diameter) under aseptic conditions, and incubated at 37° for 18 hours. The plate, which then shows few or no colonics and is followed by one containing a considerable number, is taken as the end-point. In some cases it is necessary to repeat the process with larger or smaller volumes of hop extract in order to obtain an end-point. If 0.1 c.c. of hop infusion is taken to represent a preservative value of 1,000, other volumes are represented by numbers in inverse ratios to this.

It was concluded that, since within limits the concentration of the hop infusion had no effect on the results, and the preservative value fell off only slowly when the spent hops were successively re-extracted, the hops retain a portion of their activity after extraction, owing to the presence of the preservative principle as a sparingly soluble substance, or to its gradual formation during the process of extraction, or both. Its quality certainly appears to be of more importance than its quantity. The method also showed conclusively that, though the preservative power and soft resin content are associated, there is no quantitative relationship between them. The ratio of the former to the latter varied, in fact, from 1:5 to 1:50.

The B. Delbrückii Method (Hastings, Pyman and Walker, *J. Inst. Brewing*, 1926, 32, 484): This method, which had already been used by Windisch, Kolbach and Rathke (*Woch. Brau.*, 1924, 41, 175 *et seq.*), was suggested in a modified form partly as a substitute for the

old *Bact. X.* method and partly as a standard by means of which Chapman's biological method could be directly compared with Ford and Tait's gravimetric method. It depends on the titration of the lactic acid produced by an organism in the presence of varying amounts of a hop decoction.

To 20 c.c. portions of malt wort (sp. gr. 1.040, pH 5.4–5.5), contained in plugged boiling tubes and sterilised for 10 minutes at 100° on 3 successive days, are added varying volumes of a 0.25% hop decoction, prepared from 4 grm. of minced hop boiled for 1 hour with 1,600 c.c. of water, in a 2-litre flask, on a sand-bath. The volume of the cooled decoction is again made up to 1,600 c.c., and the supernatant liquid pipetted off. Quantities from 0.5–5.0 c.c. are usually required (differing by 0.5 c.c.), with a tube of wort as control, and after the volumes have all been made up to 30 c.c. with water, the tubes are again sterilised for 10 minutes. One c.c. of a mixture of 2 c.c. of a vigorous 24 hours-old culture of *B. Delbruckii* in wort (obtained from daily sub-cultures for 4 successive days) and 48 c.c. of sterile wort, are rapidly added to each tube, by means of a sterile pipette, under aseptic conditions. After 40 hours at 40°, the contents of each tube are washed into a conical flask, so that the total volume is 100 c.c., and the lactic acid produced is titrated with 0.025*N* sodium hydroxide solution, with phenolphthalein as indicator. After the titration of the control is allowed for, that volume of hop decoction which completely suppresses growth, (*i. e.*, produces "zero" titration) is in reciprocal relationship to the antiseptic power.

Bacterium Delbruckii is stated by Jørgensen ("Micro-organisms and Fermentation," 1925) to ferment cane sugar, dextrose, lævulose and maltose, but not lactose, with the production of lactic acid. It grows vigorously in unhopped wort or maltose-broth media at 35–52°, (optimum range for acid production 40–48°).

The same paper contains a description of investigations on the behaviour of this organism under varying conditions, and a critical discussion and comparison of the three methods described in this section.

The Gravimetric Method.—This is based on the original work of Ford and Tait (*J. Inst. Brewing*, 1924, 30, 426) and has been the subject of some discussion and modification, the reasons for which have already been mentioned. The following final method, described by Walker (*ibid.*, 1928, 34, 9), is stated to give good results.

The freshly mixed sample (10 grm.) is extracted in a 100 c.c. Soxhlet apparatus with ether (sp. gr. 0.730) on a water-bath at 55°, for 3 hours. The residual resin, after removal of the ether by distillation, is extracted with successive 20 c.c. portions of redistilled methyl alcohol (b. p. 65°) at 50–60°, the combined extracts made up to 100 c.c. at 15° and filtered through a dry paper, and the α -resins in 40 c.c. of the filtrate precipitated at 60° with a fresh 1% solution of lead acetate in methyl alcohol. An excess of reagent greater than 2 c.c. should be avoided, and the end-point is determined by the transference of a drop of the supernatant clear liquid to a filter-paper moistened with sodium sulphide. It should be noted here that the brown lead sulphide stain which indicates an excess of lead, may also be produced by a trace of lead salt if any is accidentally transferred with the liquid on the rod. After 30 minutes the lead salt is filtered off on a tared Gooch crucible, washed with methyl alcohol, dried at 100° for 1 hour and weighed. The factor 15.75 gives the percentage of α -resin.

The total soft resin is determined on 20 c.c. of the same filtrate, diluted with 40 c.c. of a 1% solution of sodium chloride, and extracted 4 times with 50 c.c. of petroleum spirit (b. p. below 40°). The combined filtered extracts are evaporated in a tared flask, and the residue dried at 100° for 1½ hours and weighed. The β -resin is then found by difference. The dilution operation is important (see p. 125), as under the above conditions the extract obtained is a maximum and the results then conform with the expression $(\alpha + \beta/3)$ for the preservative value. This has been confirmed independently by Burgess and Martin (*J. Inst. Brewing*, 1928, 34, 13).

The α -resin determination may be shortened by the exact measurement of the volume of lead acetate solution required for complete precipitation. The factor 0.2325 then gives the percentage of α -resin, with an accuracy of 0.2%. Ford and Tait (*loc. cit.*) state that if there is any doubt as to its purity, the lead salt may be ground up with dilute sulphuric acid and alcohol, extracted with the petroleum spirit, and a solution in methyl alcohol of the washed residue, after evaporation, reprecipitated or titrated. In a subsequent paper Hastings and Walker (*id.*, 1929, 35, 229) indicate that cheaper grades of methyl alcohol may prove suitable for the extraction, and describe a more rapid method, in which the resin is extracted directly in 3 minutes in a stirring apparatus. Windisch, Kolbach and

Winter (*Woch. Brau.*, 1929, **46**, 101 *et seq.*) have also modified the method.

Other methods of less importance have also been suggested for the measurement of antiseptic value. On the Continent, in particular, a method is used in which the stability of the beer brewed from a particular hop is determined. This, of course, gives a result in accordance with the demands of practice, but as a method is difficult to standardise, and is subject to many outside influences.

Van Laer (*Pet. J. Brass.*, 1926, **34**, 254; 1928, **36**, 790) and Hastings, Pyman and Walker (*loc. cit.*, p. 127), made a comparative study of the above three methods and obtained results in good agreement for a number of hops. The main conclusion of the latter workers is that the chemical (gravimetric) method is to be recommended, since it is most easily standardised. Biological methods, the *Bact. X* method in particular, may give variable results due to changes in the virility of the organisms, though the results are obtained more quickly, and less working time is required than with the gravimetric method. This applies particularly to Chapman's method, whilst a disadvantage of the *B. Delbruckii* method is that it does not measure the power of the hop to suppress both multiplication and functional activities of the organism. It is apparent, however, that if a gravimetric method is used in practice, final appeal must always be made to a biological method, against which the former can be standardised.

Future work will tend towards the investigation of a standard antiseptic substance, in terms of a given weight of which the preservative value of a sample of hops may be expressed, (*cf.* the Rideal Walker test). Pure humulon has been suggested, and also salicylic acid (Brown and Clubb, *J. Inst. Brew.*, 1913, **19**, 272), though Chapman (*id.*, 1929, **35**, 363) has recently shown that a 1% solution of phenol is most suitable for the purpose and gives very constant results over long periods of time. Ten c.c. mixtures of nutrient agar and 0.5–2.5 c.c. of phenol solution are sterilised for 10 minutes, cooled to 40°, 3 drops of an 18 hours broth culture of the organism (grown at 37°) added, and the mixture plated out and incubated at 37° for 48 hours. The organism grows freely in the presence of 1.5 c.c. but not of 2 c.c. of phenol solution.

Other problems of the future are the investigation of the change in the properties of the hops during the brewing processes, and their preservative value in the finished beer.

Of more practical interest are the results of a comparison of the values determined by the above methods with the stabilities of the beers produced in brewing trials with the hops, and with the personal judgment of a panel of experts obtained by hand inspection of the sample (Lones and Grant, *J. Inst. Brew.*, 1927, **33**, 6; Thompson, *id.*, 564). Though the correspondence is not perfect, (British Columbian hops being a notable exception), a very fair approximation has been obtained, particularly in comparison with Chapman's method. The older methods of examination by "rub," on the other hand, were found to be very reliable, and there is much to be said for their cultivation (see p. 143). Unfortunately they are confined to the expert. Large quantities of hops are still bought on the basis of hand examination alone.

Changes in Antiseptic Value on Storage, Etc.—This is a matter of some importance in the assessment of the value of a sample of hops. Hops are usually stored in the dark, in cool, dry places and under conditions which minimise chances of oxidation. When they are to be kept for long periods refrigeration is used (Armstrong, *4th. International Congress on Refrigeration*, June, 1924), and a certain amount of deterioration then occurs which has been measured by Chapman (*loc. cit.* p. 126) in terms of the changes in soft resin content and preservative value. His results show that cold storage merely retards the changes associated with ageing. The falling off in soft resin content is initially slightly greater for ordinary hops than for hops from cold store, but this difference is less marked as time proceeds. The preservative value falls off more quickly than the soft resin content at first, and then remains constant, while the latter still decreases. The decrease is least marked for the cold-stored hops. Experiments on a 30-year old hop from cold store (*J. Inst. Brew.*, 1927, **33**, 574) confirmed these results; the α -acid had almost completely disappeared, and the whole of the little preservative power which remained was due to the β -resin.

Chapman (*id.*, 294) also found that green, freshly picked hops had a far higher preservative power before they were kilned and dried, though no increase occurred during the ripening process. According to Hastings and Walker (*id.*, 1928, **34**, 556) this may be due to the presence of crystals of lupulon in the β -portion of the soft resin which would otherwise be destroyed by heat. In addition, the work of Burgess (*id.*, 1928, **34**, 248; 1929, **35**, 235) has demon-

strated that the colour and aroma of the hops decrease with increase in drying temperature, though sterilisation by heat and storage out of air is preferable to drying, from the point of view of retaining antiseptic value. There is obviously much information to be obtained on the optimum conditions of drying from a detailed study of kiln design. The seasonal variations are discussed by Hind and Meyer (*J. Inst. Brew.*, 1924, 30, 725).

Finally, Chapman (*loc. cit.*) concluded that the high preservative value found for hops which had been highly compressed for packing purposes was not necessarily due to the inhibition of oxidation, nor to rupture of the seeds and removal of the fatty oil during extraction, but more probably to the break-up of the strobiles under pressure, and the production of a finer state of division of the hop.

Soft Resins.—The soft resin-content, apart from its bearing on the preservative power, is a valuable analytical number, and the valuation of hops on this basis, which was customary before the publication of the above methods, will no doubt continue. Chapman (*6th Congress of Industrial Chemistry*, Paris, 1927) emphasises this, and points out that the soft resin content, considered in conjunction with the amount of hard resin, gives a good indication of the general richness and degree of maturity of the hop.

Of the numerous methods suggested for the determination, those of Walker and of Chapman are probably to be preferred in the light of recent knowledge of the hop. The former has already been described (p. 128), and Chapman's improved method (*loc. cit.* p. 126) follows:—

The hops (10 grm.) are allowed to soak for 12–15 hours at 20° in 400 c.c. of ether (sp. gr. 0.730), and then at the b. p. of ether for 5–6 hours. The solution is made up to 507 c.c. at 15° (7 c.c. are occupied by the hops), filtered rapidly, and 100 c.c. of filtrate, measured in a tared flask, evaporated and the residue dried in the steam oven till constant in weight. This gives the total resins. For the soft resins, a further 100 c.c. of filtrate are evaporated to a small volume, and spread evenly on an Adams milk analysis paper supported in a horizontal position. The last traces of resin may be transferred to the paper with a little ether. The dry paper is coiled, extracted in a Soxhlet apparatus with petroleum spirit (b. p. 45–55°) for 6 hours, the filtered extract evaporated, and the residue dried in a steam-oven in a tared flask, till the weight is constant.

This method, which has been found to give consistent and accurate results for many varieties of hops, is an improvement on those previously described, in that the hops are not extracted with petroleum spirit directly. On account of the poor powers of penetration of this solvent, low results are otherwise obtained. The results obtained by the new method are lower than those obtained by Walker's methyl alcohol method, but the author considers them to be more accurate. It is also claimed that the method is simpler and requires only one extraction of the hop, whilst the solvents used are cheaper and more easily recoverable than methyl alcohol.

Titration of the resins has also been suggested, and in the method of Walker (quoted above) it is combined with the gravimetric determination. A rapid method is that of Winge and Jensen (*Compt. rend. Trav. Lab. Carlsberg*, 1914, **11**, 116), in which it is assumed that the greater bitterness of the soft resin compared with the hard is compensated by the greater equivalent of the latter.

The hops (5 grm.) are transferred to a 300 c.c. flask and dried in a vacuum for 24 hours at 35° C. The dried material is then covered with 150 c.c. of absolute ether, left for 1 hour and repeatedly shaken. The liquid is then filtered, and the residue washed with 100 c.c. of ether. Finally, the filtrate is titrated with a 0.05*N* alcoholic (93%) potassium hydroxide solution, with 6 to 8 drops of 1% phenolphthalein as indicator, the titration being continued until further additions of alkali no longer increase the colour intensity. Each c.c. of 0.05*N* potassium hydroxide solution corresponds with 0.02 grm. of resin. This factor is exact only for the soft resin; the hard resin has a much higher equivalent, but, as the bitterness of the resins is approximately proportional to the reciprocals of their equivalents, the use of this factor approximately measures the bitterness of the hops. It is essential to use dry hops and absolute ether, as otherwise tannins pass into solution and introduce errors. Even with hops containing so little as 4.5% of water, and with absolute ether, the resins may be over-estimated by 1% (on the hops).

Heintz (*Woch. Brau.*, 1925, **42**, 325) shakes 10 grm. of hops for 2½ hours with 250 c.c. of chloroform, and titrates 100 c.c. of the filtered solution (in the presence of 50 c.c. of neutral 96% alcohol, to avoid an emulsion) with an alcoholic solution of potassium hydroxide, and with phenolphthalein as indicator. The indistinct end-point which is produced by the coloured solutions is overcome by the use

of a control, which consists of an alkaline solution of phenolphthalein compensated by a similar hop extract.

Moisture.—This is carried out in many laboratories by drying 3 grm. of the hops *in vacuo* over sulphuric acid. If the necessary apparatus is available, the method is a good one, but almost identical results may be obtained by drying in a steam-oven, a minimum weight being reached as a rule in 3 hours or less. Those who prefer the use of a vacuum desiccator usually base their preference on the loss of essential oil with the moisture at the temperature of the steam-oven. But some, at least, of the essential oil is given up even in the cold *in vacuo*, and the percentage of essential oil is very small in relation to that of moisture and certainly less than the unavoidable error of sampling. Winge (*Woch. Brau.*, 1915, 32, 45) states that the hop resins remain unchanged, and complete desiccation is obtained after 24 hours *in vacuo* at 35°.

The drying process, as usually conducted in England, reduces the moisture in hops from 60 or 70% to less than 6%. By subsequent exposure on the packing floor, the moisture always rises somewhat, and, if the time of exposure is long and the atmosphere very moist, it may rise to 12% or more. Such hops do not keep well, and hops containing more than 11% of moisture should not be bought except for immediate use and, of course, only if suitable in other respects. On the other hand, hops containing much less than 8% of moisture are inclined to be brittle and scarcely bear handling without the risk of falling to powder. Not only does this breaking up imply risk of loss of lupulin, but the brewer prefers whole hops for other reasons.

Detection of "Sulphuring."—Some confusion exists on this subject in the text-books, which use this term indifferently to designate two entirely different practices. In Great Britain a certain quantity of sulphur is usually thrown on the fire of the drying kilns towards the end of the drying process. The resulting sulphur dioxide tends to restrict the subsequent development of moulds and other objectionable micro-organisms, though the concentration of the gas in the atmosphere of the kiln can hardly destroy all the spores. So far as it acts as an antiseptic, its action is of course useful, but the grower's chief motive for the use of sulphur on the kiln is the improvement it effects in the appearance of his hops. If this misleads the brewer in his choice of hops, the practice is not free from objection, but English brewers are good judges of hops, and possibly the antiseptic

value of sulphur dioxide outweighs the objection attaching to the production of a fictitious appearance. Brewers here and there attribute occasional yeast difficulties to the use of heavily sulphured hops, but the evidence of causal connection is seldom very strong when investigated. However, sufficient prejudice exists in certain quarters for requests to be made to the analyst to determine whether or no a particular sample of hops has been sulphured, and some Continental works devote considerable space to the subject.

Prior (*Chemie u. Physiologie d. Bieres*, Leipzig, 1896) gives the following directions for the detection of sulphuring:—The hops (10 grm.) are extracted in the cold with 200 c.c. of distilled water in a large flask, and the mixture is frequently shaken during half an hour, at the end of which time it is filtered. Of the clear filtrate, 50 c.c. are brought into a 150 c.c. Erlenmeyer flask, together with 1.5 grm. of zinc and 25 c.c. of hydrochloric acid (sp. gr. 1.125); sodium amalgam may be used if the zinc is likely to contain sulphur. The mouth of the flask is loosely plugged with filter paper, of which the portion facing down the neck of the flask is moistened with a solution of basic lead acetate. At the end of half an hour the plug is removed and examined. According to Prior, the presence of only 1% of freshly sulphured hops in the sample is sufficient to give rise to a distinct brownish-yellow coloration of the plug under the conditions of the test. This is said to correspond with at least 0.07 mg. of SO_2 in the 50 c.c. of hop extract (= 2.5 grm. hops) used, or 0.003% on the sample. The prescribed conditions must be strictly adhered to, since, if the evolution of hydrogen is too violent, most hops will yield hydrogen sulphide (Lintner, *Chem. Ztg.*, 1908, 32, 1068).

If several reduction experiments are made with the same hop extract and the several plugs examined after 3, 5, 8, 10, etc. minutes respectively, an approximate measure of the extent of sulphuring may be obtained. A distinct discoloration within 5 minutes is said by Prior to correspond with 0.012% of SO_2 in the sample, or if within 3 minutes, with 0.15%. Heavily sulphured hops, fresh from the kiln, may contain as much as 0.3% SO_2 , but under ordinary storage conditions at least half of this disappears within a month, and in the course of very few months falls to 0.07%, at which it remains fairly stationary for at least a year. Sulphites and free sulphur dioxide may be determined, if necessary, by the methods usually employed in connection with the detection of preservatives (Cf. Monier-

Williams, *Analyst*, 1927, **52**, 343, 415; 1928, **53**, 118). A suitable weight of hops is boiled with 300 c.c. of air-free distilled water, and 10 c.c. of concentrated hydrochloric acid, in a current of carbon dioxide, and the sulphur dioxide conveyed through a condenser into an excess of hydrogen peroxide, by means of an adapter dipping below the surface of the liquid. The distillate (about 250 c.c.) is acidified with hydrochloric acid, and the oxidised sulphites precipitated in the cold with barium chloride, as barium sulphate. Allowance should be made for the sulphate content of the hydrogen peroxide used.

Preservation of hops by sulphur dioxide has been attempted by Burgess (*loc. cit.*, p. 131). Since the use of this gas produced a greater reduction in preservative value than an equal amount of gas obtained by burning the equivalent weight of sulphur, it is not likely to be greatly used, in spite of the improved colour it imparts to the hops. Burgess considers that about 3% of the sulphur is retained by the hops and may be converted into lead sulphate in the examination by the gravimetric method (p. 128).

Detection of Free Sulphur.—The other practice referred to as “sulphuring” is that of dressing the growing hops with flowers of sulphur to arrest the development of mould. This is often the only available means of saving the crop, and no argument like that sometimes directed against the use of sulphur in the kiln, that it is a practice the benefit of which is wholly on the side of the grower, can be brought against it, for lost crops mean high prices which hit the brewer. But it has been said that, when hops have been heavily sulphured in the field, their use in the brewery has given rise to “stench,” owing to the sulphur finding its way to the fermenting vessels and, in contact with living yeast, being reduced to hydrogen sulphide. It is possible for sulphur, as such, to find its way on to the hops during the kilning process also, if the sulphur used in that process is unskillfully applied.

A rough qualitative test is obtained if a silver pin, stuck in the pocket of hops, becomes tarnished. For the detection of sulphur in hops, according to the so-called Burton method, 5 grm. of the hops are heated with 250 c.c. of water and 5 grm. of slaked lime in a beaker, on a water bath for 20 minutes. A portion of the solution is filtered, cooled and tested at once with a freshly prepared dilute solution of sodium nitroprusside. Nearly all English hops, when tested in this

manner, give a red coloration. An experienced worker may decide from the intensity of the colour whether the hops can properly be described as containing notable or excessive amounts of free sulphur, but the chemist turning to this book for information will presumably not have experience of hops and their sulphur content. The coloration is transient and could not easily be made the basis of a quantitative method with any pretensions to accuracy, but solutions for comparison could be made up from sulphur-free hops and weighed amounts of sulphur. The necessary sulphur-free hop is more likely to be found among a collection of Oregons or Bavarians than among the English samples which may be available.

For the quantitative *determination of free sulphur* in hops, the following method is preferable: The hops are extracted with xylene in a Soxhlet or other extractor, a piece of bright copper foil (reduced in hydrogen) having been first placed in the flask and weighed with it before adding the xylene. The xylene dissolves the sulphur, which at the temperature of boiling xylene combines with the copper to form copper sulphide, which in small quantity is fairly adherent. A dozen siphonings are sufficient. The foil is washed a few times by decantation with small quantities of xylene, then with petroleum spirit, and the flask dried in a steam-oven, cooled and weighed. The increase in weight gives the amount of sulphur in the hops taken for the experiment. Large quantities of copper sulphide tend to flake off and might be lost in washing, but hops seldom, if ever, contain enough sulphur to give rise to flaking.

Arsenic.—English hops almost invariably contain detectable traces of arsenic derived from the fuel used for drying them. The Royal Commission on Arsenical Poisoning, in its final report issued in 1903, recommended that the Board of Inland Revenue should prescribe for the different materials used in the preparation of beer an adequate test by which their freedom from arsenic might be ascertained. The Commission further expressed the opinion that no substance should be used as a food, or as an ingredient of food, which contained more than $\frac{1}{100}$ grain of arsenic per pound or per gallon, according to whether it was a solid or a liquid. It was at first argued by hop merchants that the recommendation of the Commission was unreasonable when applied to hops, which are used in relatively small quantities in beer, namely, 1 to 5 pounds per barrel. If the hops were the sole source of arsenical contamination, therefore, even

the use of 5 pounds of hops containing $\frac{1}{20}$ grain of arsenic per pound would only introduce $\frac{1}{4}$ grain of arsenic into the barrel, or much less than the allowable $\frac{1}{100}$ grain per gallon. But hops are not the sole source of arsenical contamination. Malt, the principal raw material of the brewer, always contains detectable traces of arsenic, and it is not reasonable to require the maltster to deliver it with less than $\frac{1}{500}$ grain per pound, though he frequently succeeds in doing so. Brewing sugars, too, are not quite free from arsenic, though in those of British manufacture the amount is, as a rule, extremely minute. Since in strong ales as much as 3.5 pounds of malt may be used per gallon, the use of malt containing $\frac{1}{500}$ grain of arsenic per pound might introduce as much as 0.007 grain of arsenic into each gallon. If there is to be certainty that the finished beer shall not contain more than 0.01 grain per gallon, not more than 0.003 grain must be allowed to find entrance with the other materials. Such a strong ale will probably be heavily hopped at the rate, say, of 5 pounds per barrel or 0.14 lb. per gallon, and it will be clear that the hop used should not contain more than $\frac{1}{50}$ grain of arsenic per pound. It is fortunately true that a variable, but usually notable, proportion of the arsenic introduced with the materials is eliminated by the yeast, but the brewer needs to be on the safe side. If he compelled the maltster to guarantee less than $\frac{1}{300}$ grain of arsenic in his material, it seems unreasonable to try to stiffen this guarantee in order to free hop growers from the obligation of conducting their kilning operations with reasonable care. For the extent of arsenical contamination can be kept down by careful selection of fuel and by proper application of sulphur to the kilns if sulphur must be used. When coal is burned, some of the arsenic is volatilised, but some—with many fuels, the larger proportion—remains in the ashes. It has been shown by Baker and Dick (*J. Soc. Chem. Ind.*, 1904, 23, 174) that, when sulphur is thrown on the fire, the arsenates in the ash may be reduced, with the result that from two to four times as much arsenic may be volatilised and reach the goods on the kiln as is the case when sulphur is not used. Voelcker (*J. Roy. Agric. Soc.*, 1923, 84, 357) has shown that hot air kilns cause less arsenical contamination of the hops than open fires, and since special stoves for burning the sulphur apart from the coal fire are now also available, there seems no excuse for hop growers who market hops with more than ten times as much arsenic as is commonly found in malt.

It may be admitted that the Commission's recommendation of $\frac{1}{100}$ grain, as applied to hops, is a counsel of perfection rather than a standard to be rigidly enforced, and $\frac{1}{50}$ grain per pound represents a maximum that may be permitted with safety.

The estimation of arsenic in hops is carried out by means of the Marsh-Berzelius method (Vol. I, page 195) or its modification. In such cases, 10 grm. of unground hops are well stirred with 100 c.c. of boiling distilled water and 1 c.c. of concentrated sulphuric acid in a 500 c.c. beaker for 20 minutes. The extract is filtered through cotton-wool, evaporated to 10 c.c., and transferred to the Marsh apparatus. If organic matter is not previously destroyed, the arsenic may be under-estimated, but it is the practice in many brewing laboratories to omit this tedious procedure. Opinions differ on this point, however, (see Chapman, *Analyst*, 1913, 38, 599), and in some cases, particularly in the presence of a high percentage of sulphur, the aqueous extract obtained by the above method, and not the whole sample, is oxidised. The brewer's object is to exclude highly arsenical consignments and, provided he is right in assuming that at least two-thirds of the arsenic is recovered when omitting the previous destruction of organic matter, he avoids risk of serious contamination of his beer, and any complaint addressed to the hop merchant has an added weight if it is communicated to him that the analytical method on which the complaint is based is one that cannot over-estimate and may considerably under-estimate the amount of arsenic actually present.

For the destruction of organic matter, Thorpe (*J. Chem. Soc.*, 1903, 83, 974) moistens 5 grm. of the hops with lime-water, adds 0.5 grm. of lime or magnesia, mixes thoroughly, and then heats over a low Bunsen flame until the organic matter is completely charred, and finally in a muffle, until practically all the carbon is burned off. When cold, the ash is moistened with water, 20 c.c. of sulphuric acid (1:7) added, the dish warmed, and its contents transferred to a flask. About 0.5 grm. of potassium metasulphite is added, and the solution boiled until free from sulphurous acid. When cool, the whole or an aliquot portion of the solution is transferred to the electrolytic Marsh apparatus. The deposit obtained is compared with a series of standards prepared in a similar way from arsenic-free (air-dried) hops and measured volumes of a suitably diluted solution of arsenious oxide.

From their bulky nature, it is impossible to moisten hops except by the use of a large quantity of lime-water, and it is also very difficult to secure adequate mixture with the lime or magnesia. The acid method for the destruction of organic matter is, therefore, preferable.

The hops are placed in a 300 c.c. Kjeldahl flask with a suitable volume (about 10 c.c.) of arsenic-free sulphuric acid, concentrated nitric acid added, 1 c.c. at a time, and the mixture well boiled. Care should be taken that the mixture does not go dry, and this is avoided if an excess of nitric acid is ensured. When the solution is colourless or no further change in colour is observed, the solution is cooled, diluted with an equal volume of water, and again concentrated to remove the nitric acid. The solution is again diluted and transferred to the Marsh apparatus. If less than 5 gm. of hops are taken, the quantities of acid recommended above may be reduced, but the use of 5 gm. enables a more representative sample to be obtained and, if the amount of arsenic in the hops is so large as to give rise to mirrors not easily compared with the standards, the final solution may be made up to 50 c.c. and an aliquot portion taken for the test.

Tannin.—Formerly some few chemists and brewers attached great importance to this estimation, but Chapman (*J. Inst. Brew.*, 1907, 13, 646; *id.*, 1909, 15, 360) has demonstrated beyond doubt that the tannin of hops plays a much less important part in the brewing process than was at one time supposed and that the estimation of its amount, in the present state of our knowledge, gives no guide as to the brewing value of a particular sample. For this reason the consideration of the estimation of this natural constituent of hops has been postponed until after due account has been taken of such commonly occurring impurities as free sulphur and arsenic. The estimation is still asked for by some brewers, however, on the grounds that a high percentage of tannin indicates a good normal hop that is not too old, and that the tannin serves a useful purpose as a precipitant of albuminoid matters (chiefly albumoses) in the brewer's copper, and as a means of imparting a distinctive bitterness to the beer. That hops introduce almost as much soluble nitrogenous matter into the wort as they remove from it had been pointed out by earlier workers (*e.g.*, Briant and Meacham, *J. Fed. Inst. Brew.*, 1897, 3, 482) and has been demonstrated more exactly by Chapman (*loc. cit.*). Chapman has also shown that there

is no causal connection between age and percentage of tannin, and there is no evidence of any such connection between percentage of tannin and the brewing value of hops.

Methods for the determination of tannins are discussed in Vol. V, page 106 *et seq.* It should be added that all early work on hop tannin is vitiated by the method used to ascertain its amount, usually a modification of the Loewenthal method (Heron, *J. Fed. Inst. Brew.*, 1896, 2, 162), the best method in its day, but one which, in addition to other disadvantages, assumes that the whole of the substances removed by the gelatin is tannin. If the determination is to be made, it should be made by Chapman's method (*J. Inst. Brew.*, 1907, 13, 646) which does rest on scientific principles.

Ten grm. of the hops are placed in a flask marked at 508 c.c. (8 c.c. = estimated volume of 10 grm. of wet hops); 400 c.c. of boiling distilled water are added and the hops are macerated with the aid of a stout glass rod, suitably bent at the end. The flask with its contents is then immersed for 2 hours in a water-bath, which is kept gently boiling. The contents of the flask are cooled, made up to 508 c.c. with cold distilled water at 15°, and filtered. Fifty c.c. of the clear filtrate are evaporated slowly in a small beaker on the boiling water-bath. If the extraction and concentration are conducted as described, no trouble will be experienced from conversion of the hop tannin into a reddish, insoluble substance, to which the name "phlobaphen" has been given, but in regard to which little is known beyond the fact that it is readily formed when solutions of hep tannin are boiled and that this fact must be considered in connection with any method for the estimation of hop tannin. When the 50 c.c. of clear extract have been concentrated to about 15 c.c., they are cooled and the tannin precipitated by addition of 50 c.c. of a saturated solution of cinchonine sulphate (about 1.5%). After standing for 1 or 2 hours the precipitate is filtered off on asbestos in a Gooch crucible. In preparing this, the asbestos should be washed with a 0.5% solution of cinchonine sulphate, pumped as dry as possible and dried to constant weight in a steam oven. The precipitate of cinchonine tannate is poured into the Gooch crucible and allowed to filter, at first without the aid of a pump. When about half the liquid has run through, suction is applied, and the whole of the precipitate transferred to the crucible. When all the liquid has filtered through, the precipitate, which is much less

soluble in cinchonine sulphate solution than in water, is washed several times with a 0.5% solution of cinchonine sulphate. Care should be taken not to allow the precipitate to dry on the sides of the beaker, as it then becomes difficult to remove. When washing is completed, suction is continued until the cake of precipitate is moderately dry, as shown by its tendency to separate into several portions. The crucible, with its contents, is then dried to constant weight in a steam oven.

Chapman has shown that the compound of cinchonine with gallo-tannin contains 63% of its weight of gallotannin, corresponding with 2 molecules of gallotannin to one of cinchonine. The nitrogen content of the precipitate obtained from hop extracts corresponded with 42% of cinchonine, the balance of the precipitate (58%) being, therefore, hop-tannin. Chapman, accordingly, proposes to multiply the weight of cinchonine tannate found by 0.6 and to return the result as hop-tannin. In carrying out the determination in the manner described, 50 c.c. of extract, corresponding with 1 grm. of hops, are taken, and the weight of cinchonine tannate found, multiplied by 60, gives the percentage of tannin in the hops.

Ling and Nanji (*J. Inst. Brew.*, 1921, 27, 310) also recommend Chapman's method, but introduce a modification which enables the determination to be made more rapidly. The hop is extracted in the usual way, and to 100 c.c. of extract, evaporated to 80 c.c., are added 50 c.c. of a 1% solution of cinchonine sulphate, clarified if necessary by the addition of a drop of sulphuric acid. Since this cinchonine sulphate solution has an optical activity represented by a reading of 4.92° V. through a 1 dm. tube ($[\alpha]_D$ for cinchonine = $170.3 - \text{Landolt}$),¹ the amount of cinchonine sulphate precipitated by the tannin in the hops may be calculated with an accuracy equal to that of Chapman's method.

Mitchell (*Analyst*, 1923, 48, 14; 1924, 49, 167) has applied his ferrous tartrate and osmium tetroxide reagents for the determination of tannins (see Vol. III, 566; V, 187) to the case of hops. The latter provides a simple and rapid method if Chapman's gravimetric method is taken as a standard of comparison. The cinchonine reagent precipitates true tannin (less colouring material), and good agreement between the two methods is obtained if the factor 2.65 is used to convert the colorimetric results into lupulo-tannin.

¹ The factor for the conversion of $^\circ$ V. into degrees of arc for the sodium D-line is 0.3459.

Physical Examination of Hops.—The importance of hand examination has already been emphasised (p. 131), and is supported by the fact that cases have been cited where examination by an expert has yielded results in a few minutes in disagreement with, but often more reliable than a series of lengthy and elaborate chemical experiments, (Windisch Kolbach and Banholzer, *Woch. Brau.*, 1926, 43, 79). In Great Britain hops are bought for the most part by brewers as a result of hand examination, or, as it is said, are judged by the “nose” and “rub.” In order to form a fair judgment of hops as a result of such an examination considerable experience is required, which nothing written in a book can replace. But the main points to which a brewer directs his attention may be briefly stated.

To begin with, the brewer likes a whole hop and a sticky hop. When hops are broken up, there is danger that the glands may be ruptured and their resinous contents set free, when deterioration, both in loss of aroma and conversion of the resins, is accelerated. The cones, also, should be single and not bunched, and small fragments on the faces indicate that the hop is unsuitable for “dry-hopping.”

Good, fresh samples of individual hops, rich in lupulin, stick together and only slowly open out again when pressed tightly in the hand, although, as a whole, a properly dried sample is very elastic and springy. If the stems are darker than the corresponding strobiles, an absence of “sulphuring” is indicated.

An examination of the glands with a lens is useful. In fresh, well-managed hops, these are of a golden yellow colour, with slightly wrinkled surfaces and oily contents which readily ooze out under pressure. With age they become more wrinkled and darker in colour, and finally assume a deep red-orange tint. The strigs should be free from moisture and the lupulin may be seen adhering in the bracts. High drying induces changes in appearance similar to those which naturally result with age. In general, the hop should chaff finely when rubbed down, and leave no residue of fibrous matter, but only a yellowish resinous deposit on the fingers.

Some brewers show a decided preference for a hop with very few seeds and, other things being equal, this is reasonable, for the seeds weigh heavy and are useless to the brewer. It has been shown by Salmon and Amos (*J. Inst. Brew.*, 1908, 14, 309) that certain of the

finest English varieties of hops, notably Goldings, do not grow out well unless a fair amount of fertilisation is allowed to take place, so that any general aversion to seeded hops, such as is shown by German brewers, would probably lead to the disappearance of these varieties as unprofitable to the grower. Their brewing value, however, is such that they will doubtless continue to be bought up readily in spite of the presence of many seeds. Burgess (*J. Inst. Brew.*, 1925, 31, 623) also, has pointed out that the seed content of a sample of hops is a varying factor, and one which depends on chance. It must therefore be allowed for where accurate determinations of the resins or other constituents are required, particularly as the seeds contain upwards of 25% of a fatty oil which may be extracted if any become crushed. The weight of hop due to the seeds is determined on a 5 grm. sample of whole hops, of known moisture content, from which the strigs and bracts are removed, and the remainder rubbed to a powder. This is sifted through a 20-mesh sieve, the residue re-rubbed and re-sifted, and the final residue placed on a plush-covered tray ($7 \times 30 \times 2$ inches) inclined at an angle of 20° . This is tapped gently, when the seeds fall to the bottom, and are collected, dried at 100° , and weighed.

There is a growing disposition to admit that, in the past, the brewer has given too much attention to the delicacy of colour of hop samples. A good sample should be bright in colour, a dull, rusty appearance being generally an indication of a damaged hop, but a brown-coloured hop is not necessarily objectionable, nor will it give increased colour in the copper if the brownness be due merely to ripeness. An exceptionally pale colour probably indicates that the hop was picked while still unripe. This does not necessarily mean that the preservative resins will not be fully developed and some brewers in fact show a decided preference for pale hops. The colour of the foliage leaves which may be found in the sample affords a guide to the manner in which drying has been conducted. These leaves which should not be present in the hop sample itself should be of a fresh, lively green colour, and not dirty brownish or olive-green. The colour of a hop depends principally upon its origin, and the following method for its estimation may prove useful in particular cases. Netscher (*Petit. J. du Brass.*, 1928, 36, 86) adds alcohol to a filtered hop decoction, and thus precipitates the tannins, proteins, and calcium phosphate, which remove about

as much colour as the peptones and tannins precipitated in practice in the copper. A determination of colour on the filtrate is then possible. The change in colour with time of the liquid from yellow-green to brown-yellow is least for a good quality hop. The higher the pH value of the medium, the darker is the colour of the hop.

The aroma of hops is best judged after rubbing some of the sample on the back of the hand, and many insist that the hand should be that of the person who is passing judgment. Since the quantity of oil in the hop is very small (0.2 to 0.5%) and, the differences in different samples are due apparently to variations in the proportions of the minor constituents, it will be obvious that the chemist's task would be a very difficult one, even if he knew which of the minor constituents contributed to the delicacy or rankness of odour which distinguishes hops of different origin and growth. As a matter of fact, we have not yet this complete knowledge, and the nose of the buyer remains his sole guide in the matter of aroma. Here, therefore, more than in any other part of the hand examination, is experience essential and written instruction almost useless. The brewer desires, not merely to satisfy himself that the hops possess sufficient aroma of the right kind, but that they are free from objectionable odours, (or odours which in his experience connote deterioration due to age or damp packing), or a flavour which will overpower the delicacy of other hops with which they may be used. Damp packing gives rise to a musty odour, whilst old hops acquire an odour not unlike that of cheese, and due probably to valeric acid. A slight trace of a sweet smell similar to heated clover hay indicates over-heating in the drying process.

The practical brewer derives a good deal of information from an examination of the "side" of the cube cut out by the sampling knife, but enough has been written on a subject which is essentially one calling for practical demonstration by an expert provided with a varied collection of hops.

The method of classification and assessment of hops as a result of physical examination is a matter of personal preference. The three divisions chosen by Wetherall (*J. Op. Brewer's Guild*, 1920, 6, 29) are useful and include (1) Nicely cured and mould-free hops; (2) hops which show traces of mould; (3) under- or over-cured hops, which show considerable mould, and cannot be stored, but may be used in the copper.

On the Continent a possible 100 marks are awarded to each sample, and the total is made up as follows:—Lupulin (24), aroma (24), cone development (20), colour and lustre (10), sorting (10), dryness (6), and picking (6).

Detection of Hop-substitutes in Beer

Whatever may have been the case formerly, this subject has very little importance at the present time, in Great Britain at all events, and that for three reasons.

In the first place the proportion of hop-substitutes used in Great Britain is infinitesimal. Brewers are required to record in the Excise books all the materials they use, and the returns for recent years show that the practice is decreasing. Thus, in 1914 174 cwts. of hop *substitutes* were used in the United Kingdom, whilst for 1923 and 1927 the figures for Great Britain and Northern Ireland are 37 and 34 cwts., respectively. Hop *preparations* (see p. 148), however, increased from 42 to 98 cwts. between 1923 and 1927. It may be said that some of the possible substitutes possess so intensely bitter a taste that they would displace more than an equal weight of hops. On the other hand, it is a fact that a large proportion of the so-called hop-substitutes are manufactured from hops, more than one patented process being worked, having for its object the separation of the preservative and flavouring constituents of the hop and the use of these by the brewer at different stages of his process, (see p. 148). Papers by Gautier and Guerin (*Ann. Brass. et. Dist.*, 1912, 15, 241) and by Schönfield (*Woch. Brau.*, 1915, 32, 25) may be cited in this connection.

In the second place, even if hop-substitutes found more extensive use, it is not the business of any official in Great Britain to detect the use of such substitutes, there being no law as to what the brewer may or may not put into his beer, beyond the provision of the Food and Drugs Acts that these must be declared and that no substance which is injurious to health may be added to any food intended to be offered for sale.

In the third place, the brewer's own permanent interests forbid him to make use of hop-substitutes, and statistics show that, for the most part, he recognises this. The bitter of hops differs from all other vegetable bitters in its fugitive character, since substitutes usually consist of quassia, chiretta or powdered barks, and sometimes

aloes, mixed with hop-powder. When the bittered liquid has left the palate, the bitter taste passes also and does not continue to affect the palate as do other bitters, in some cases for a long time after the liquid in which they were dissolved has been swallowed. Beer drinkers as a class may not know this distinction between the bitter of hops and other bitters, but there is abundant evidence that they often have very sensitive palates, and it may be asserted with confidence that, guided by their palates alone, they would quickly abandon the use of beer bittered with any notable quantity of hop-substitute.

Allen devoted a great deal of his time and exceptional skill to the detection of hop-substitutes, as may be seen by reference to a file of the *Analyst*, but with the diminishing importance of the subject it

OUTLINE PROCESS FOR THE DETECTION OF BITTER PRINCIPLES IN BEER

One litre of beer is evaporated to half its bulk and precipitated boiling with neutral lead acetate, the liquid boiled for fifteen minutes and filtered hot. If any precipitate occurs on cooling, the liquid is again filtered.

Precipitate contains <i>hop-bitter</i> , <i>caramel-bitter</i> , <i>ophelic acid</i> (from <i>chiretta</i>), phosphates, proteins, etc.	Filtrate. The excess of lead is removed by passing H_2S , and the filtered liquid concentrated to about 150 c.c. and tasted. If any bitter taste is perceived, the liquid is then slightly acidified with dilute sulphuric acid, and shaken repeatedly with chloroform.	
Chloroform layer on evaporation leaves a bitter extract in the case of <i>gentian</i> , <i>calumba</i> , <i>quassia</i> , and <i>old hops</i> (only slightly or doubtfully bitter in the case of <i>chiretta</i>). The residue is dissolved in a little alcohol, hot water added, and the hot solution treated with ammoniacal basic lead acetate and filtered.	Aqueous liquid is shaken with ether.	
Precipitate contains <i>old hops</i> , <i>gentian</i> , and traces of <i>caramel</i> products. It is suspended in water, decomposed by H_2S , and the solution agitated with chloroform.	Ethereal layer leaves a bitter residue in the case of <i>chiretta</i> , <i>gentian</i> , or <i>calumba</i> . It is dissolved in a little alcohol, hot water added, and the hot solution treated with ammoniacal basic lead acetate and filtered.	Aqueous liquid , if still bitter, is rendered alkaline and shaken with ether-chloroform. A bitter extract may be due to <i>berberine</i> (<i>calumba</i>) or <i>strychnine</i> .
Chloroform layer is examined by special tests for <i>gentian</i> and <i>old hop-bitter</i> .	Filtrate is boiled to remove ammonia, and treated with a slight excess of sulphuric acid filtered and tasted. If bitter, it is agitated with chloroform, and the residue examined for <i>calumba</i> and <i>quassia</i> .	The aqueous liquid , separated from the ether-chloroform, may contain <i>caramel-bitter</i> or <i>choline</i> .
	Precipitate is treated with water and decomposed by H_2S . The filtered liquid is <i>bitter</i> in presence of <i>gentian</i> .	Filtrate is treated with a slight excess of dilute sulphuric acid, filtered and tasted. A bitter taste indicates <i>calumba</i> or <i>chiretta</i> , which may be reextracted with ether and further examined.

will suffice to mention that the tannin figure (p. 140) may sometimes be a guide to the presence or absence of these substitutes, and to give the foregoing table from among the eight pages devoted to the subject in a previous edition.

The table calls for one or two comments. Modern preparations of *caramel* do not, as a rule, yield any precipitate with lead acetate, and a note of warning should be issued with regard to the mere detection of *quassia*. When it is remembered that quassia may be used by hop growers, like other gardeners, to combat aphids, and that large quantities are, in fact, so used in hop gardens in Oregon and California which send large quantities of hops to the British market, it will be obvious that the mere detection of quassia in beer by a very delicate test affords no certain evidence that the brewer has knowingly made use of quassia as such.

Aloes are also sometimes used and are detected in the residue from 200 c.c. of the evaporated beer, by the addition of warm ammonia. The aloe-resin is then precipitated in the cool, filtered liquid, by hydrochloric acid, and is identified by its taste and odour etc., (see p. 79).

As far as concerns substitutes for hops intended to supplement its preservative value, reference may be made to the recent Public Health (Preservatives etc. in Food) Regulations, 1925, (in force from Jan. 1927) in which 70 parts per million of sulphites (as sulphur dioxide) but no other preservatives, are permitted in beer. This quantity more than compensates for the preservative powers of the largest amount of hops likely to be used by the brewer. For its determination in beer reference may be made to *Analyst*, 1927, 52, 343, 415; 1928, 53, 118.

Hop oil and hop extracts are more properly classed as hop adjuncts than as hop substitutes, since they are obtained from the hop and are intended to supplement certain of its constituents. The former, however, is often sold in an adulterated state, and the detection of foreign materials is a matter of some difficulty. The specific gravities, boiling-points, refractive indices and optical dispersions of the fractions obtained by distillation under 15–20 mm. pressure may be ascertained, together with their relative proportions, aroma and solubilities in alcohol, (see Chapman, *loc. cit.*, p. 119). The oil is usually added in small quantities to the finished beer in the cask (*cf.* "dry-hopping"). This compensates for the loss of the oil

from the original hops during the boiling, and is thus particularly useful where open coppers are employed.

A number of patents cover the production of hop extract or concentrates. The raw material may be extracted with boiling water under pressure, or with hot alcohol, and sometimes a mixture of water and alcohol vapours is used. The filtered liquid is concentrated by evaporation under reduced pressure, and hop oil, obtained from the original material by steam-distillation, is added to the concentrate. Such preparations often have high resin-contents and preservative powers, and these may be determined by the methods described above for the hops themselves. In the former case a known weight of sample is dissolved in a little water and/or alcohol and spread on an uncoiled Adams milk analysis paper, which is then dried in warm air, coiled and extracted in the manner described on p. 132. Hop extracts are usually added to the wort before it enters the copper, and sometimes towards the end of the boiling process. A good product may contain about 20% of petroleum spirit-soluble ("soft") resin.

BIBLIOGRAPHY

None of the books on this subject contains the whole of the recent analytical work described above. Reference may be made however, to the *Journal of the Institute of Brewing* from about 1920 onwards, and for other aspects of the subject the following publications may be consulted.

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ENZYMES

BY JULIUS GRANT, PH.D., M.Sc., A. I. C.

The detection and relative estimation of enzymes is in most cases a matter which requires some experience. This is due partly to the difficulty of obtaining enzyme preparations in the pure state, particularly from complicated materials such as plant and animal tissues, but principally to the fact that so many outside conditions influence enzyme reactions. These include:—

(a) Temperature. The reaction velocity increases with the temperature up to a maximum point and then decreases to zero.

(b) Concentration of the substrate.

(c) Concentration of the enzyme. This should be proportional to the rate of change of the substrate in methods used for the estimation of the activity of the enzyme.

(d) End-products of the reaction.

(e) Hydrogen ion concentration (pH value).¹ One or more optimum values exist for each enzyme according to its origin.

(f) Inorganic salts or organic compounds which inhibit or accelerate, either temporarily or permanently, enzyme action. These are often specific in action, but may usually be removed from the system.

Thus in the investigation of extracts of leaves or plant material for enzymes, the influence of tannin, which renders the enzymes inactive, must not be overlooked. Brown and Morris (*J. Chem. Soc.*, 1893, **63**, 604) have shown that the comparatively weak diastatic action of some leaves is due to the tannin they contain. E. F. Armstrong has found this to apply to emulsin and other enzymes, including the oxydases. Tannin also interferes with the determination of sugars by Fehling's method, and should be precipitated from the leaf extract by means of basic lead acetate, the solution filtered, the volume of the filtrate noted, and the lead precipitated by hydrogen sulphide. The precipitate is filtered off,

¹ See *The Control of Reaction in Cultures and Enzymic Digests*. F. W. Foreman and G. S. Smith, Special Rept. No. 32, of the Dept. of Scientific and Industrial Research, 1928.

washed, the filtrate evaporated to remove hydrogen sulphide, and the residue made up to the original volume. Maubert (*Ann. Brass. et Dist.*, 1929, **27**, 280) has shown that many enzyme reactions are stimulated by the α -particle constituent of radioactive sources but inhibited by the β - and γ -rays.

Enzymes enclosed in tissues with a living cell wall cannot, as a rule, be extracted until the protoplasmic structure is destroyed. This may be effected in a number of ways:

(1) The material is dried quickly at a low temperature, 20–30°, and, if necessary, when partly dry is warmed to 50° or more. It is then treated with water, and the filtered solution precipitated with alcohol.

(2) By autolysis of the material, usually with the addition of an antiseptic to prevent bacterial action.

(3) By rapid dehydration effected by stirring with absolute alcohol or acetone.

(4) By mechanical disintegration of the living tissue. This is effected in a Buchner press or by grinding the cells in a mortar with sand. Soft organisms, such as bacteria, can be frozen hard with liquid air and then ground. The thick paste produced is placed in a hydraulic press, and the enzyme-containing liquid filtered through a clay candle to remove the non-ruptured cells.

In general, aqueous or glycerol extracts of the materials, prepared in some such manner as described, are used as sources of enzymes. In special cases the solid material is used directly. The extracts may be purified from crystalloids by ordinary or electro-dialysis. The dissolved enzyme can be precipitated with alcohol or acetone, washed with alcohol and ether, and dried in a vacuum over sulphuric acid, and is thus obtained as a soft colourless powder. Protein impurities may usually be removed by adsorption on filter-paper from an aqueous solution (J. T. Wood, *J. Soc. Chem. Ind.*, 1918, **37**, 313). The paper is dried rapidly at a low temperature to fix the proteins, and then soaked in water for a short time to remove the enzyme. For colloidal solutions Michaelis and Davidson (*Biochem. Z.*, 1924, **144**, 294) adjust the *pH* of the solution to the iso-electric point, and then precipitate the enzyme. Such processes generally entail a loss of activity.

Enzyme preparations, particularly those which are not dried, required to be preserved. A few drops of toluene will form a surface-

layer over the liquid, and, although this substance injures pepsin, trypsin and lipase in the course of time, it is to be preferred for general purposes. Chloroform, though easily mixed with the solution, is soon lost by evaporation, but a mixture of equal parts of it with toluene, used in a 1% concentration, is very suitable. Sodium fluoride (0.3%), ether, phenol, thymol, mustard oil and formaldehyde are also used in particular cases, but are all more or less injurious to the enzyme.

Since enzymes are measured by their activities, it is of fundamental importance that the amount of enzyme shall be proportional to the change produced. The numerous investigations on the kinetics of enzyme action have established that this relation holds only when the amount of enzyme is relatively small, compared with the amount of the substance on which it acts, and further, that the change must not exceed the conversion of about 30 or 40% of the original substance. The latter part of this statement, which virtually includes the first part also, is commonly referred to as Kjeldahl's law of proportionality, and illustrates the catalytic nature of enzyme action. Kjeldahl stated originally, that the amount of reducing sugar formed by the action of malt extract upon an excess of starch was a true measure of the diastatic power, so long as the digestion was not carried beyond the point corresponding with a 40% conversion of the starch, and that up to this point the velocity curve could be expressed by a straight line.

The limitations of Kjeldahl's law were further defined by Schütz (*Z. physiol. Chem.*, 1885, **9**, 577; 1900, **30**, 1) and extended to the digestion of proteins by pepsin. Deviations from the typical curves of catalysis may be due to inhibiting impurities, to the effects of the reaction-products, or to the combination of the enzyme with the substrate. The simple law of mass action, therefore, cannot be applied (Hedin, *Z. physiol. Chem.*, 1925, **146**, 122). The concentration of substrate at which half the maximum velocity is attained is a measure of the affinity between the enzyme and substrate (Haldane, *Nature*, 1928, **121**, 207). According to Berkson and Flexner (*J. Gen. Physiol.*, 1928, **11**, 433) the course of enzyme reactions involving changes in the viscosity of the medium are represented by the equation $V_t - V_w = (V_f - V_w) / \{1 - [(V_0 - V_f) / (V_0 - V_w)]\} e^{-rt}$, where V_w , V_0 , V_t , and V_f are the rates of flow of water, and of the enzyme-substrate mixture initially, after the time

t, and at the end of the reaction, respectively. See also Aberhalden and others, (*Fermentforsch.*, 1929, 10, 319 et seq.).

Enzymic activity may be expressed as: (1) The amount of change produced by a given amount of enzyme in a given time, (2) the amount of enzyme required to produce a given change in a given time, or (3) the time required for a given amount of enzyme to effect a given change. In addition, the conditions referred to above must be maintained constant, and the substrate must be either chemically pure or available in quantities sufficient for a large number of comparative experiments. Hard glass apparatus should be used. The neglect of these precautions may lead to altogether false values.

Attempts to obtain general expressions for the activity of enzymes have not been wholly successful, but reference may be made to those of Willstätter and Kuhn (*Ber.*, 1923, 56, [B], 509) and of von Euler and Josephson (*id.*, 1926, 59, [B], 770). Von Euler and Svanberg (*Z. physiol. Chem.*, 1919, 106, 201; 1921, 112, 193) suggest units which are an absolute measure of the activity of the enzyme in terms of the velocity-constant of the unimolecular reaction it promotes. At present, however, it is preferable to express the activity of each enzyme in units best suited to the investigator, method and enzyme concerned.

The methods for the measurement of enzyme activity depend on (1) the formation of reaction products which may be identified or determined, (2) the disappearance of the substrate, (3) changes in the physico-chemical properties or appearance of the system, owing to the changes included in (1) or (2). An example of the fourth type which may be applied to any enzyme reaction in which there is no evolution of gas or volatile vapours is the micro-method of Sastri (p. 173). Different methods will not give the same absolute values of the activity when applied to the same enzyme preparation, since they measure different effects and do not take equal account of side-reactions, the presence of accelerators or paralyzers, or of other enzymes. They should, however, enable a number of preparations of the same enzyme to be classed in the same order of relative activity, and in all cases the results should be reproducible.

For the present purposes enzymes will be classified according to Waksman and Davison (see Bibliography) as follows:—

I. HYDROLYTIC ENZYMES.

- (1) Esterases. (Lipase, lecithinase, esterase, phosphatases, phytase, sulphatase.)
- (2) Carbohydrases.
 - (a) Polysaccharidases. (Diastase, inulase, pectinase.)
 - (b) Trisaccharidases. (Gentianase, raffinase.)
 - (c) Disaccharidases and Glucosidases. (Invertase, maltase, lactase, emulsin, tannase.)
- (3) Acting on proteins.
 - (a) Coagulating. (Thrombin, rennin.)
 - (b) Proteases. (Pepsin, trypsin, anti-trypsin, enterokinase, pancreatin, erepsin, plant proteases.)
 - (c) Amidases. (Arginase, urease.)

II. OXIDISING, OXIDO-REDUCING ENZYMES and ZYMASES.

- (1) Oxydases. (Plant oxydases, peroxydase, tyrosinase.)
- (2) Oxido-reductases. (Schardinger's enzyme.)
- (3) Reductase.
- (4) Zymases. (Zymase, carboxylase.)

III. CATALASE.

ESTERASES

Lipase (Steapsin).—The preparation and general properties of lipase are described by Haley and Lyman, (*J. Amer. Chem. Soc.*, 1921, 43, 2664). Castor-oil seeds are shelled, freed from oil and powdered; the milk is separated and allowed to stand 24 hours, when an emulsion rises to the surface. A portion of this is triturated with olive oil and a trace of manganese sulphate, and the formation of fatty acids measured by titration. Castor oil lipase is insoluble in water and reacts in acidic solutions. Other lipases are allowed to act on ethyl butyrate, and the acid formed determined by titration or by the conductivity method. Pancreatic lipase is tested with neutral olive oil or with an aqueous emulsion of egg yolk, and the free acid formed in either case titrated.

The saponifying effect of lipase is demonstrated by the blue-green colour produced after 10 minutes, when a saturated solution of copper sulphate is added to a mixture of the enzyme with equal portions of 2% agar and 5% starch solutions plated-out for 1 hour at 55°. An increase in acidity may be shown by comparison of boiled and unboiled mixtures to each of which brom-cresol purple is added.

In the Kanitz test (*Z. physiol. Chem.*, 1905, 46, 482) 10 c.c. of olive or castor oil (neutralised to phenolphthalein) are incubated for 10–24 hours with 2–5 c.c. of lipase extract, with a boiled solution of enzyme as control. The free fatty acids produced are a measure

of the activity and are determined by titration with 0.1*N* sodium hydroxide solution to phenolphthalein neutrality in the presence of 50 c.c. of neutral 96% alcohol and 5 c.c. of neutral ether. After subtraction of the control titration figure, the acids (as oleic acid) are given by $28.16 \times$ volume required. Alternatively, the fatty acids may be extracted with ether and titrated. Palmer (*J. Amer. Chem. Soc.*, 1922, **44**, 1527) prepares an emulsion (fat-content 4% and *pH* 5.0) from acacia powder (15 grm.), melted butter (20 grm.) and warm distilled water ground together in a mortar. The emulsion (75 c.c.) is incubated with 1 c.c. of lipase for 24–48 hours, and 25 c.c. titrated to phenolphthalein neutrality with 0.1*N* alcoholic potash solution in the presence of acetone (75 c.c.) and ether (25 c.c.). Similar methods are used for **Lecithinase**.

Rona and Lasnitzki (*Biochem. Z.*, 1924, **152**, 504; 1926, **174**, 373) place a mixture of dilute lipase preparation with an equal volume of Ringer's solution¹ in a sealed tube attached to a manometer, and spill into it 0.3 c.c. of tributyrin from a separate compartment. The volume of carbon dioxide evolved by the action on the bicarbonate of the butyric acid produced, may then be measured. Demuth (*Biochem. Z.*, 1924, **150**, 392; 1926, **174**, 373) has suggested a stalagmometric method which requires the removal of any casein by coagulation with rennin.

The *phyto-lipase unit* is the amount of enzyme solution required to hydrolyse 7.5% of 2.5 grm. of olive oil (saponification value 185.5), at *pH* 4.7 (produced by 2 c.c. of a 0.5*N* acetate buffer), in 20 minutes (Willstätter).

The activity of lipase, which is a maximum at *pH* 5.0, is increased by bile-salts, lecithin and cholesterol, and decreased by fluorides (especially at low *pH* values) and arsenites (Corran, *Biochem. J.*, 1929, **23**, 188).

Esterase.—Bach and Zoubkoff (*Compt. rend.*, 1920, **171**, 967) detect esterase in blood by the action for 30 minutes at 20° of 1 c.c. of a dilute solution (1:1000) on 0.2 grm. of potassium sulphoguaia-colate in 7 c.c. of water, with 1 c.c. of dilute peroxydase solution (1:20), and 1 c.c. of 1% hydrogen peroxide. A control experiment is necessary. The guaiacol liberated by the esterase is detectable by its reaction with the peroxydase (see p. 185), which is itself inert towards phenolic esters.

¹ An aqueous solution of 9 grm. of sodium chloride, 0.25 grm. of potassium chloride, 1.5 grm. of sodium bicarbonate and 0.2 grm. of calcium chloride per litre.

A good quantitative method is that of Falk (*J. Amer. Chem. Soc.*, 1913, **35**, 601; 1915, **37**, 217, 649), in which the butyric acid liberated by the enzyme from a neutral 1% solution of ethyl butyrate after 24 hours at 37° is titrated with 0.05*N* sodium hydroxide solution.

A simple and satisfactory physical method is that of Dale and Evans (*J. Physiol.*, 1920-1, **54**, 167). The time to produce a pH of 7 is determined from the colour change of 8 drops of a 0.02% solution of phenol red in a mixture of 5 c.c. of a fresh 1% solution of glycerol triacetate, 5 c.c. of a phosphate buffer, and 0.5 c.c. of a 0.3% solution of sodium glycocholate with 0.3 c.c. of the sample. A control is necessary, and the end-point is gauged by comparison with a colour standard. Rona and Michaelis (*Biochem. Z.*, 1911, **31**, 345) measure the increase in surface tension during conversion, from the decrease in the number of drops of enzyme-substrate mixture (pH 8.0) falling from a pipette under standard conditions.

Phosphatases are classified by Lüers and Malsch (*Woch. Brau.*, 1929, **46**, 143, 153) according to the nature of the organic phosphate combinations they decompose. Thus *glycerophosphatase*, *nucleotidase*, *phytase* and *saccharophosphatase* have optimum activities at 36°, 49°, 48° and 41.5°, and pH 5.2, 5.6, 5.2 and 6.0, respectively. They are obtained principally from barley or from bones, and in the latter case they may be extracted in chloroform water for 7-10 days, the proteins precipitated from the extract at pH 5.8, and the solution dialysed. They are identified by the methods of Forrai (*Biochem. Z.*, 1924, **145**, 48, 54). Glycerophosphatase, which decomposes glycerophosphoric acid into glycerol and phosphoric acid, is estimated from the gravimetric determination of the increase in the phosphate-content of a mixture of sodium glycerophosphate, toluene, and the enzyme, incubated for 24 hours at pH 5.4-6.0. The proteins should be precipitated with sodium chloride, kaolin and 2 drops of acetic acid. **Phytase** is measured in a similar fashion, (Collatz and Bailey, *J. Ind. Eng. Chem.*, 1921, **13**, 317), or from the increase in electrical conductivity of a phytin-phytase mixture.

Sulphatase is measured in terms of the decrease in organically-combined sulphate it produces (Neuberg, *Naturwiss.*, 1924, **12**, 797 and *Biochem. Z.*, 1926, **174**, 457).

CARBOHYDRASES

Diastase (Amylase).—This enzyme is technically of the greatest importance and is the one most often examined in analytical practice.

An active diastase is prepared by the precipitation of a malt extract with alcohol, and pancreatin, malt diastase and taka-diastase are official pharmaceutical preparations. The methods of estimation used include (1) the measurement of the maltose produced by saccharification of soluble starch by the enzyme; (2) the estimation of the power of the enzyme to form products which do not give a coloration with iodine solution; and (3) measurements of physical changes in the system.

As a qualitative test for diastase in honey the A.O.A.C.¹ recommends the digestion of 10 c.c. of a threefold diluted solution of honey with 1 c.c. of a 1% solution of soluble starch at 45° for 1 hour. The colour produced with 1 c.c. of a dilute solution of iodine is then compared with that of an unheated control. A heated or artificial honey gives a blue colour, whilst the presence of active diastase is shown by a green or brown colour. Quantitative methods for use with honey have been standardised by Fiehe and Kordatzki (*Z. Unters. Lebensm.*, 1928, **55**, 162) and criticised by Lampitt, Hughes, and Rooke (*Analyst*, 1929, **54**, 381). The method finally adopted by the latter workers is a modification of that of Ohlsson (*Compt. rend. Trav. Lab. Carlsberg*, 1926, **16**, 1) in which two enzymes are determined separately, viz.—(a) *Dextrinogen-amylase*.—Tubes containing 1 c.c. of 0.2% starch solution, 8 c.c. of phosphate buffer (pH 5.6), and 1 c.c. of honey solution of varying concentrations are heated at 38° for 30 minutes, cooled in ice, and 2 drops of 0.02*N* iodine solution added. If *W* and *W'* are the weights of honey in the boundary tubes between brown and purple (for dextrin and reducing sugars), or between purple and blue (for starch and dextrin) the corresponding diastatic activity is $0.5 (2/W + 2/W')$.

(b) *Saccharogen-amylase*.—Flasks containing 25 c.c. of 2% starch solution, 10 c.c. of phosphate buffer (pH 5.6) and 10 c.c. of 4% honey solution are heated at 38° for 30 minutes, 1 hour and 16 hours, a similar mixture containing honey solution boiled for 15 minutes serving as control. The contents are then cleared with 10 c.c. of alumina cream, made up to 100 c.c., filtered, and the sugar content of the filtrate determined. The diastatic power is the number of mg. of maltose produced.

The orders of the activities are about the same for the two enzymes, but both decrease rapidly if the honey is heated above 70°.

¹ "Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists." 1925.

Lintner Methods.—Lintner's method is described in connection with malt, together with the modification of Ling recommended by the Institute of Brewing (Vol. I, pp. 176-9). These important methods have been the subject of much criticism. In particular, the outside indicator recommended by Ling is troublesome. The change of the colour of the Fehling's solution from blue-green to yellow may be gauged more accurately with the naked eye of the practised worker so long as re-oxidation of the copper is minimised by quick working. The methylene-blue or dianol-green indicators of Lane and Eynon (*J. Soc. Chem. Ind.*, 1923, **42**, 32 T) and J. S. Mann (*Chem. and Ind.*, 1926, **4**, 187, 545), respectively, are much to be preferred. The method of preparation of the soluble starch, also, will not always yield a product which gives the same results. The nature and control of the changes which occur during the preparation process are not fully understood, but the work of Gore (*J. Amer. Chem. Soc.*, 1925, **47**, 281) has shown that the *pH* value should fall between 4.5-5.6, whilst Fabre and Pénau (*J. Pharm. Chim.*, 1923, **28**, 289, 341) state that alkalinity activates pancreatic diastase, but inhibits malt diastase, and acidity has the reverse effect. Astruc and Renaud (*id.*, 1923, **27**, 333) suggest that the washed starch should be dried so as to contain 7-8% of water and passed through a No. 100 sieve. Hind, Threadgold and Arnold (*J. Inst. Brew.*, 1926, **32**, 26) accordingly recommend the production of *pH* 4.6 by the addition to the solution of 20 c.c. of a buffer solution containing 500 c.c. *N* acetic acid and 68 gm. of crystalline sodium acetate per litre. The diastatic conversion is arrested by means of 20 c.c (instead of 10 c.c.) of 0.1*N* sodium hydroxide solution. The optimum *pH* at any given temperature (*t*) is given by the expression $(82.5 + t)/25$, but it does not follow, however, that an adjustment of the *pH* alone will necessarily ensure a reliable starch. Thus, Ohlsson (*Compt. rend. Trav. Lab. Carlsberg*, 1927, **16**, No. 7, and *loc. cit.*, *supra*) states that malt diastase contains two enzymes, which act together but have different optimum *pH* values. Taka-diastase is most active at *pH* 4.5-6.5.

Sherman, Kendall and Clark (*J. Amer. Chem. Soc.*, 1910, **32**, 1073, 1087; 1928, **50**, 2529 *et seq.*) also found that commercial preparations of pancreatic diastase are too poor in electrolytes to function normally when caused to act upon pure starch dissolved in pure water. Neutral salts are essential, and anions particularly chlorine ions,

are, in general, more effective than cations. In their improved method 0.3 grm. of sodium chloride and 7 c.c. of 0.02*N*/ disodium hydrogen phosphate are added to 100 c.c. of 2% starch solution. Digestion takes place at 40°, in vessels already at this temperature, for exactly 30 minutes, after which period 50 c.c. of Fehling's solution are added, the flask immersed in a bath of boiling water for 10 minutes, and the reduced copper determined by any of the usual methods. The weight of cuprous oxide must not exceed 300 mg., and is corrected from the value found in a blank without the enzyme. By plotting the velocity curve of diastatic action, which is not a straight line, with times as abscissæ and yields of reducing sugar as ordinates, a scale is obtained which permits of an expression of true diastatic power based on the weight of cuprous oxide to be obtained. If 300 mg. of cuprous oxide are taken as 100 on this scale, then the corresponding scale values of *K* are given in the following table:

Cuprous oxide		Cuprous oxide		Cuprous oxide		Cuprous oxide	
Mg.	K	Mg.	K	Mg.	K	Mg.	K
30	9.1	100	31.2	170	54.1	240	78.3
40	12.2	110	34.4	180	57.5	250	81.8
50	15.3	120	37.6	190	60.9	260	85.4
60	18.4	130	40.9	200	64.3	270	89.0
70	21.6	140	44.2	210	67.8	280	92.6
80	24.8	150	47.5	220	71.3	290	96.3
90	28.0	160	50.8	230	74.8	300	100.0

The values of *K* are divided by the respective weights of enzyme. When any other time is used as standard, 30 minutes are divided by the actual time in minutes and multiplied by the value of *K*. Thus in 30 minutes 0.15, 0.30, 0.45, 0.60 mg. of pancreatin yielded, respectively, 76, 147, 217, 286 mg. of cuprous oxide, whence $K = 23.5, 46.5, 70.2$ and 94.8 ; then the diastatic powers are 156, 155, 156, 158 units, respectively. More accurate results, however, may be obtained by elimination of organic matter if the precipitate is oxidised to cupric oxide by heat and then weighed according to the usual method employed for reducing sugars.

In the case of flour the sample is usually prepared by Rumsey's method (Fairbrother, *Indust. Chem.*, 1929, 5, 313) 10 grm., being digested with 100 c.c. of water at 27° for 1 hour, and the suspension diluted to 175 c.c. and clarified with 3 c.c. of 15% sodium tungstate solution adjusted to about pH 2 (thymol blue) with sulphuric acid. The reducing sugars may then be determined on 50 c.c. of the extract diluted to 200 c.c. and finally clarified in a centrifuge. The blank obtained from a similar solution obtained without digestion for 1 hour is deducted. A constant rate of reaction is ensured by Ambard (*Bull. Soc. Chim.*, 1921, 3, 51) who states that diastase can be removed from solution to the extent of 96–100% by crude starch-powder. The enzyme is then removed from the filtered starch complex in a solution of sodium chloride at pH 6.60.

Engelhardt and Gertschuk (*Biochem. Z.*, 1926, 167, 43) have devised a micro-method, based on the micro-determination of sugars, for use with 0.06 c.c. of blood.

Lintner's method and its modifications become unreliable for highly active malts and diastase preparations (such as those used in the textile industry), with a diastatic power greater than 50° L. In such cases the method of Davison (*J. Amer. Chem. Soc.*, 1920, 42, 293) or of Windisch and Kolbach (*Woch. Brau.*, 1925, 42, 139) may be used. In the former the starch (100 grm.) is dyed with neutral red (0.5%), and washed free from the excess of dye. The determination of the end-point in Lintner's method is then easily detectable. In the latter 100 c.c. of starch solution (buffered with 5 c.c. of a solution of 27.21 grm. of sodium acetate and 512 c.c. of *N* acetic acid per litre) are digested with 5 c.c. of a 4% enzyme preparation for 30 minutes at 20°. The action is stopped with 3 c.c. of 3*N* sodium hydroxide solution, and the solution made up to 200 c.c. and titrated with 0.1*N* iodine solution (*vide infra* Baker and Hulton).

Iodine Methods.—Roberts' method (*Proc. Roy. Soc.*, 1881, 32, 145) has been modified by Johnson (*J. Amer. Chem. Soc.*, 1908, 30, 798), Sherman, Kendall and Clark (*id.*, 1908, 30, 798) and others, and is a rapid, though less accurate, method. The time taken at 40° to convert a 1% starch solution into achroodextrin, the achromic point or first point at which no colour is given by iodine solution, is measured. The time taken (the chromic period)

must be between 4 and 10 minutes, failing which, the quantity of enzyme solution must be suitably altered.

To 5 c.c. of a 1% solution of soluble starch with 2 c.c. of 0.5% sodium chloride solution and 2 c.c. of a phosphate buffer of pH 6.71 at 40°, are added 1, 2 or 3 c.c. of the diastase solution. At half-minute intervals a drop is removed, tested with a dilute solution of iodine and the time (t) noted when no blue colour is produced. The diastatic power is given by $5n/vt$, and is the number of c.c. of starch solution (n) converted by 1 c.c. of enzyme solution (diluted to a volume v) in 5 minutes.

In Wohlgemuth's method (*Biochem. Z.*, 1908, 9, 1; 1909, 21, 432) from 0.1 to 1.0 c.c. of enzyme solution and 0.5 c.c. of toluene are added to 5 c.c. portions of a 1% solution of starch contained in test-tubes. After 30 or 60 minutes or 24 hours at 38–40°, the action is stopped by cooling the tubes to 0°, the liquids diluted, and 1 drop of a 0.1*N* solution of iodine added, when a range of colours from dark blue, blue-violet, reddish-yellow to yellow is obtained. The violet colour indicates complete hydrolysis to dextrin. The diastatic power is expressed as the number of c.c. of starch converted by 1 grm. of enzyme (or 1 c.c. of its solution) at 40° in the period of time chosen. The method is applicable over a wide range of diastatic activities. The method of Baker and Hulton (*Vol. I*, p. 136) also is accurate and convenient except in cases where reducing sugars other than maltose are produced (*e. g.*, with taka-diastase), but a correction should be made for iodine absorbed by the starch and malt extract (*cf.* Windisch above). Cohen and Dodds (*Brit. Med. J.*, 1924, I, 618) match the colour of the iodine against that of a standard in the presence of ammonium sulphate.

The use of Mett's method (see pepsin) with starch paste as substrate is not satisfactory.

Physical Methods.—*Polarimetric Methods.*—In Gore's improved method (*Ind. Eng. Chem.*, 1928, 20, 865) soluble starch is prepared by digesting 1 part of potato starch and 1.5 parts of 13% hydrochloric acid for 6 days. The filtered residue is washed, neutralised with ammonia, washed free from chlorides, and adjusted to pH 4.8 by the addition to 100 c.c. of 6% starch solution of 2 c.c. of a solution 100 c.c. of which contain 8 c.c. of *N* acetic acid and 12 c.c. of *N* sodium acetate solution. The rotation of a filtered mixture of 50 c.c. of starch solution, and 5 c.c. of a 5% filtered malt infusion

is then determined, (a) in the presence of 1 c.c. of concentrated ammonia, and (b) after such a period at 21° that the polarisation has decreased not more than 11.3° V. (3.92° angular) in a 4 dm. tube, the ammonia being added just before the reading is taken. If D is the decrease in polarisation due to 250 mg. of sample in t hours, l the length of the tube in dm., and c ($= 4.6$) a constant determined from a standard starch and malt of known properties, then the diastatic power in °L, is $100 D/t.l.c.$ Myers and Killian (*J. Biol. Chem.*, 1917, **29**, 179) centrifuge the converted solution with 1 grm. of dry picric acid and read the rotation of the clear top layer.

Glycogen has also been used as substrate, in which case the unchanged glycogen is determined after digestion, polarimetrically, nephelometrically, or by titration, (Rona and Van Ewyk, *Biochem. Z.*, 1924, **149**, 174).

In Davison's viscosity method (see Bibliography) a starch solution (2-6%), boiled in the autoclave under a pressure of 18 lbs. and filtered through muslin, is prepared with a viscosity 2-3 times that of water. The pH is adjusted to 5.6 with a 0.1 M phosphate buffer. Ten c.c. of the fresh starch and 0.1-0.4 c.c. of the enzyme preparation are placed in each of two Ostwald viscometers. The times of flow of 5 c.c. at 34° are compared at intervals for the boiled and unboiled solutions. Wolff (*Chem. Ztg.*, 1915, **39**, 105) measures the difference in refractive index between two filtered mixtures of 50 c.c. of a 1% starch solution with 0.2 grm. of diastase, one of which has been boiled, and the other maintained at 38° for 2 hours. The shift in the interference bands, determined with the Zeiss refractometer, is a measure of the amount of conversion.

Diastatic actions are retarded by halogen compounds, salts of the heavy metals and formaldehyde, and alkaloids also should be extracted (Bodnár, *Chem. Zentr.*, 1916, **87**, i, 37). Fluorescein and safranin colours, potassium, toluene, chloroform, sodium and ammonium salts have an activating effect (Claus, *Biochem. Z.*, 1929, **204**, 456), while polarised and ordinary light of the same spectral composition and intensity have equal accelerating effects.

Cytase, or **Xylanase**, which hydrolyses xylan to xylose at pH 5 and 45°, is also found in germinating barley, but differs from amylase in that it is precipitated by alcohol (Lüers and others, *Woch. Brau.*, 1928, **45**, 83; 1929, **46**, 163). **Ptyalin** (saliva amylase) has been

successfully estimated by Broeze (*Biochem. Z.*, 1929, 204, 286) by the viscosity method.

Inulase. (Inulinase). The action of this enzyme on inulin for 3 days at 55° is measured in terms of the increase in copper-reducing sugars, or from the red coloration, due to fructose, produced when it is heated with resorcinol and concentrated sulphuric acid.

Pectinase, Gentianase and Raffinase (see Josephson, *Z. physiol. Chem.*, 1924, 136, 62) are measured polarimetrically or in a similar fashion to inulase.

Invertase (Invertin, Sucrase, α -Fructase) is obtained by the autolysis of yeast in the presence of sodium carbonate and chloroform. After filtration, maltase and other enzymes are removed by precipitation with alcohol. Proteins may be removed by adsorption, and the enzyme finally precipitated with 90%-saturated ammonium sulphate solution, dialysed, and adsorbed on aluminium hydroxide (Sastri and Norris, *J. Ind. Inst. Sci.*, 1928, 11, [A], 1). Aqueous solutions may be concentrated by freezing and removing the ice-crystals. The enzyme has low nitrogen and ash contents, gives no Molisch or Millon tests but positive biuret and xanthoprotein reactions. It has been identified with raffinase (see Josephson, *loc. cit.*). Von der Heide and Mändeln (*Z. Unters. Lebensm.*, 1929, 57, 13) showed that it is present in grape and wine musts and that, since its activity decreases gradually during the first five years, it is probably derived from the grapes. They measured it from the gradual decrease in rotation of added sucrose. *Taka-Invertase*, which has been obtained by Weidenhagen (*Z. Ver. deut. Zuckerind.*, 1928, 125), hydrolyses raffinose and sucrose, the optimum pH being 5.0.

Invertase is extremely sensitive to small quantities of acid or alkaline impurities, very small amounts of which may easily cause a variation in the rate of action of the enzyme to one-half of its real value. It is, therefore, absolutely necessary to make measurements under carefully standardised conditions, and on account of this the greater number of the existing determinations of the activity of invertase are valueless.

As a qualitative test, the dilute preparation and 10 c.c. of a 1% cane sugar solution are digested at 40° for 5 minutes, and the reducing power towards Fehling's solution compared with a blank which contains boiled enzyme solution. For honey the precipitate pro-

duced with alcohol may be used (Caillas (*Compt. rend.*, 1920, **170**, 589), but this is influenced by the presence of glucose and fructose (Nelson and Sottery, *J. Biol. Chem.*, 1924, **62**, 139).

Polarimetric methods are used for quantitative purposes, and that of Euler and Josephson (*Ber.*, 1924, **57**, 859) may be recommended. From a mixture of 125 c.c. of enzyme extract and 500 c.c. of a 10% cane sugar solution at 30°, 50 c.c. portions are removed at 30 minute intervals, 5 c.c. of 0.2 *M* sodium carbonate solution added (to destroy the invertase and stop mutarotation), and the optical rotation determined after 10 minutes. Proteins should be precipitated with basic lead acetate. Harding (*Chem. Zentr.*, 1922, ii, 1032) acidifies with acetic acid initially, and allows for the optical rotation of the original mixture, whilst Myers and Killian's method may also be used (*loc. cit.*).

The A. O. A. C. (*loc. cit.* p. 158) suggests a polarimetric method which is stated to be very sensitive. Twenty c.c. of a 0.5% solution of the extract and 200 c.c. of a 10% cane sugar solution, adjusted with acetic acid to pH 4.6 (*i. e.*, just acid to methyl red), are maintained at 20°. Portions are removed every 15 minutes, made just alkaline to litmus immediately, with solid sodium carbonate, and read in the polarimeter at 20°. If t is the time between the mixing and neutralisation operations, R_0 the rotation of the sugar solutions multiplied by $\frac{10}{11}$ and corrected for the rotation of the invertase solution, and R_t the polarisation after the time t , then the velocity constant of the reaction is given by

$$k = [\log_{10} 1.32R_0 - \log_{10} (R_t + 0.32R_0)] / t.$$

The mean value of k for the initial dilution should not be less than 0.1.

The micro-measurement of invertase may be made by the method of Rona and Nicolai (*Biochem. Z.*, 1926, **172**, 212), in which the carbon dioxide production of bacteria is determined after the action of the enzyme on cane sugar.

Ozone, iodine and salts of the heavy metals inhibit the action of invertase.

Maltase (α -Glucosidase) and **Lactase** (β -Galactase) may be extracted from yeast by the method of Büchner and Reischle (*Biochem. Z.*, 1917, **83**, 1). Since these enzymes convert maltose and lactose, respectively, both of which reduce Fehling's solution,

the increase in reducing power must be determined quantitatively, or Barfoed's solution (Vol. I. p. 426) (which is reduced by dextrose and not by the disaccharides) must be used. A more certain qualitative test is the preparation of the osazones (Neuberg and Saneyoshi, *Biochem. Z.* 1911, 36, 44). After the precipitation of the proteins by a solution of acetic acid and sodium chloride, the filtered solution is heated on the water-bath for 1 hour with 2 parts of fresh phenylhydrazine acetate. The laevorotary glucosazone is filtered from the hot solution, recrystallised, and the rotation read, whilst the dextrorotatory maltosazone appears in the cooled solution. The osazone solutions are best made from 0.28 gm. in 2 c.c. of pyridine, to which alcohol is added.

The increase in reducing power after incubation of a 5% solution of substrate with the enzyme, in the presence of toluene, is determined by any of the methods described in Vol. I. Pavy's method gives good results for lactose or maltose, but is less satisfactory for cane sugar (Armstrong, *Proc. Roy. Soc.*, 1904, 73, 500); proteins should be removed. One c.c. of Fehling's solution is reduced by 0.00778 gm. of maltose or 0.005 gm. of glucose. Bridel (*Compt. rend.*, 1924, 178, 1636) measures the action of maltase in malt extracts from the determination of dextrose by the emulsin method (*J. Pharm. Chim.*, 1921, 24, 81) before and after digestion in the cold for 16 days.

Kusumoto (*Biochem. Z.*, 1908, 14, 217) uses a mixture of 5 c.c. of enzyme extract with 5 c.c. of a 10% solution of the appropriate substrate, and 0.5 c.c. of toluene at 30°. Portions (2 c.c.) are removed at intervals, the proteins precipitated by 25 c.c. of a 5% solution of zinc acetate in 95% alcohol, the solution made up to 50 c.c., and the rotation read. The readings obtained in the absence of the enzyme are deducted.

Emulsin¹ (β -Glucosidase) is extracted with water from de-fatted ground almonds, and precipitated with alcohol after removal of proteins by acetic acid (Bridel and Desmarest, *Bull. Soc. Chim. Biol.*, 1928, 10, 373). It may be purified by adsorption on aluminium hydroxide and by dialysis. It is distinguished from maltase by the fact that it hydrolyses β -glucosides only. The enzyme solution (5 c.c.) may be digested for 30 minutes with 5 c.c. of a 2% solution of salicin and 1% of toluene at 40°, and the production of glucose

¹ See also Glucosides, p. 19.

and saligenin shown by the action on Fehling's solution and by the purple colour with ferric chloride, respectively.

Alternatively, amygdalin may be used as test material, and the formation of hydrogen cyanide identified with picric paper (filter paper soaked in a solution of 10 grm. of sodium carbonate and 1 grm. of picric acid per 100 c.c. and dried) which becomes a deep brick red. This test is especially suitable for plant or animal extracts which contain reducing and colouring substances (Armstrong).

The quantitative methods described for other enzymes of this group may be used for emulsin in the presence of the appropriate substrate.

Tannase.—The determination of tannase by hydrolysis of gallo-tannin is referred to in Vol. V (pp. 24, 187). Nicholson and Rhind's modification (*Analyst*, 1924, **49**, 505) of Mitchell's colorimetric method (*Analyst*, 1923, **48**, 1) gives good results. Rhind and Smith (*Biochem. J.*, 1922, **16**, 1) determine the tannin before and after the enzyme hydrolysis by titration with potassium permanganate solution in the presence of indigo-carmin.

COAGULATING ENZYMES

Thrombin (Fibrinogen) is a pseudo-globulin and gives positive reactions in the biuret, Millon, tryptophane and histidine tests. It is measured in blood by the method of Wohlgemuth (*Biochem. Z.*, 1910, **25**, 81) from the minimum amount of blood serum required to coagulate a given volume of plasma. The serum is prepared by centrifuging 3 c.c. of blood with a 33% solution of magnesium sulphate. This liquid, which keeps well in a refrigerator, is defibrinated before use, and varying volumes placed in a series of test-tubes with 2 c.c. of plasma and enough of a calcium-free 1% solution of sodium chloride to equalise the volumes. After 24 hours on ice, the tube in which coagulation is just starting is noted, and the activity of the enzyme expressed as the coagulating power of 1 c.c. of thrombin.

Rennin, a milk-clotting enzyme, found in gastric juices and in the mucous membrane of the stomach, is used commercially in the manufacture of junket and cheese; *B. prodigiosus* produces it in a medium containing complex proteins, amino acids and ammonia. It is extracted with glycerin from the fourth stomach of the calf or from the stomach of the pig, and is active only in acid solutions. It may be concentrated by adsorption on aluminium hydroxide, from

which it is removed in a phosphate buffer solution of pH 7.0. The identity of the actions of pepsin and rennin in neutral solutions has been suggested, but the results of Fenger (*J. Amer. Chem. Soc.*, 1923, **45**, 249) and of others, have disproved this. Both are proteins, but whilst pepsin is coagulated by heat and is dialysable, rennin is not. After exposure to sunlight rennin will decompose hydrogen peroxide.

Rennin in commercial preparations is measured by the amount required to coagulate a given quantity of milk in 30 minutes at 40°. Sawlajow (*Z. physiol. Chem.*, 1905, **46**, 307) allows the rennin-milk mixture to flow through capillary tubes at a constant temperature, and coagulation is indicated when the flow stops. Since ordinary milk varies very considerably in quality, it is preferable for more accurate estimations to use an artificial milk made from 3 grm. of milk powder and 27 grm. of water. The milk powder is first made into a paste with a little water, heated to 80° for 1 minute, and then cooled to 15°. The rest of the water is added, and the clear, decanted top layer, which may be kept on ice for 2 days, is used, (Blum and Fuld, *Biochem. Z.*, 1907, **4**, 62). Varying quantities of gastric juice, diluted 3,000-fold, are added to equal quantities of this milk, and the whole kept at the ordinary temperature (17.5°) for 2 hours. A drop of 20% calcium chloride solution is added, and the tubes maintained at 40° for 5 minutes. The coagulated solution to which least enzyme has been added gives a measure of the activity of the enzyme. A rough test may be first made to gauge the dilution. Morgenroth stimulates the activity of the rennin by means of a pre-digestion of the milk-rennin mixture in the presence of 1% of chloroform for 12 hours at 0°. The temperature is then raised to 38°.

PROTEASES

The proteoclastic or proteolytic enzymes may be sub-classified as: (a) Primary proteases (pepsinases), which act on proteins in acid solution; (b) Secondary proteases (trypsases), which act on denatured proteins and peptones in neutral and alkaline solutions; (c) Tertiary proteases (ereptases), which act on peptides in alkaline solutions. More correctly, primary and secondary enzymes are the only true proteases, since they act on native proteins.

Euler and Josephson (*Ber.*, 1923, 56, [B], 1749) have formulated a general expression for the activities of proteases similar to that proposed for hydrolytic enzymes and based on the affinity-constant of the reaction.

PEPSIN

Pepsin is extracted from the mucous membrane of the stomach by digestion with dilute glycerin for 24 hours. The extract is concentrated by precipitation with alcohol or picric acid, and further purified by quantitative precipitation from an aqueous solution by safranine. The dye is removed by butyl alcohol from a solution of the dye-enzyme complex in 20% alcohol and oxalic acid. Pepsin breaks down proteins into albumoses and peptones, but not into amino-acids. The optimum conditions are a temperature of 40° and pH 2. The observations of Fenger (*loc. cit.* p. 168) have enabled it to be distinguished from rennin.

Qualitative Tests.—If pepsin is allowed to dissolve fibrin dyed with carmine or rose Bengal B. in the presence of 0.2% hydrochloric acid, the extent of the reaction may be gauged from the increasing colour of the solution (see Grützner's method, p. 170). The peptonising action is followed by precipitation of albuminoids and albumoses by the tannin reagent in the presence of tartaric acid, with edestin as substrate. Pepsin gives the Molisch reaction, but neither the biuret nor xanthoprotein reactions (compare trypsin). It is inactivated by the fluorides and iodides of ammonium and potassium.

According to the British Pharmacopœia (1914, p. 285) the official pepsin is a light yellow or brown powder with a faint odour; it should dissolve 2,500 times its weight of coagulated egg-white in 6 hours. The whites of fresh eggs, boiled for 15 minutes and cooled, are passed through a hair-sieve (12 meshes per cm.), and 12.5 gm. triturated with 50 c.c. of dilute hydrochloric acid (0.65% by volume) and added to a 250 c.c. flask containing 20 c.c. of a solution of 0.25 gm. of pepsin and 1 gm. of sodium chloride in 1 litre of the acid. The mortar is rinsed with 50 c.c. of acid, and the flask maintained at 40° for 6 hours, and shaken every 15 minutes. At the end of this time the solution should be opalescent. This is a stringent test, since many pepsins of good quality will give a distinct deposit after a longer period of digestion.

The U. S. Pharmacopœia X (1925, p. 280) requires a similar test in which 20 c.c. of a solution of 0.1 gm. of pepsin in 150 c.c. of dilute hydrochloric acid, containing 12.5 c.c. of pure acid, are digested at 52° for 1½ hours with 10 gm. of sieved egg-white (No. 40 sieve), in a stoppered bottle which is inverted once every 10 minutes. The insoluble deposit is then measured by sedimentation in a graduated conical jar, and should not exceed 1 c.c.

Since, for commercial pepsins, both dissolving and peptonising powers are of importance, gastric juice is often taken as standard. On the assumption that gastric juice contains 0.45% of solids, of which 66% are inert, its "real pepsin" content is 0.15%.

Quantitative Methods.—Of the numerous substrates suggested for pepsin determinations, that of Biedermann (*Fermentforsch.*, 1917, 2, 1) which consists of the protein matter precipitated by sodium chloride from a boiling solution of egg albumin in very dilute acetic acid, made into a paste with glycerin, is perhaps most permanent and most readily digested by the enzyme. Where eggs are used, they should be 5–12 days old, since the minimum amount of undigestible matter is thus obtained. Calcified milk (10 c.c. of *N* calcium chloride solution with 50 c.c. of fresh milk) is also a standard substrate which may be used for any of this group of enzymes. Five c.c. portions, made up to 6 c.c. with the enzyme or with water, are used at 38°.

Grützner's qualitative method (see above) may be refined for quantitative purposes. Thread fibrin is soaked in ammoniacal carmine for a few seconds, dried, rinsed, macerated and suspended in the acid solution of the enzyme. The colour produced at the end of the time is matched against that of a standard calibrated against a pepsin preparation of known strength. Roaf recommends Congo red in place of carmine, as less likely to be removed from fibrin by alkali, whilst Smorodincev and Adova (*Z. physiol. Chem.*, 1925, 149, 173) consider that a 0.05% glycerin solution of diphenyl rosaniline produces a stabler complex. Magenta has also been suggested. The method is very good for small quantities of pepsin, especially if the solutions are centrifuged before the colour comparison is made.

Precipitation Methods.—The end-points of pepsin digestions may be shown in a number of ways. Voigt (*Biochem. Z.*, 1923, 142, 101) uses a 10% solution of sulpho-salicylic acid, which produces a

turbidity with urinary albumin. This is partly removed by pepsin at 38°, and the remainder estimated nephelometrically. A 1% solution of globin, precipitated from hæmoglobin, gives a flocculent precipitate with 4 drops of a 10% solution of ammonium chloride and 1-2 c.c. of 1% ammonia. A 0.25% filtered solution of pea globulin with 10% of sodium chloride and an equal volume of 0.6% hydrochloric acid is specially suitable for gastric juices. Rona and Kleinmann (*Biochem. Z.*, 1925, **155**, 34; 1926, **177**, 107; Van Arkel, *Pharm. Weekblad*, 1929, **66**, 857) measure the turbidity produced from a mixture of 7.5 c.c. of a hot saturated solution of quinidine hydrochloride and a solution of 3 mg. of casein in 5 c.c. of a 0.025*N* solution of sodium acetate (buffered with phosphates to pH 7.7). The reading must be taken within 30 minutes, and an accuracy of 2% is attainable in the absence of large amounts of sodium or calcium chlorides. Where a big coagulum is produced it may also be determined gravimetrically. In all such methods, whether the substrate is coagulated before or after the actual reaction, a control test of the turbidity produced in the former should always be made at the time of comparison.

Effront's method (see Bibliography) is quick and accurate. To 2 litres of a neutral 4% solution of egg-white, prepared by the B. P. method (p. 169), are added 20 c.c. of *N* hydrochloric acid, the mixture shaken, and filtered after 20 minutes at 80°. A 4% solution of the coagulum in 0.2% acid is then made, strained through silk, and 10 c.c. digested in a test-tube with 10 c.c. of water and a suitable quantity of pepsin. The end-point is reached when print can be read through the solution, or, after neutralisation and coagulation by acetic acid for 45 minutes in the autoclave at 100°, a nitrogen determination of the clear liquid may be made.

The Edestin Method (Fuld, *Biochem. Z.*, 1907, **6**, 473).—To various volumes of pepsin solution are added 2 c.c. of a 0.1% solution of edestin in 0.1*N* hydrochloric acid, and the end-point determined after a period at 50°, from the turbidity produced with a 10% solution of salt in 0.1*N* hydrochloric acid. Ege (*Compt. rend. Soc. Biol.*, 1922, **87**, 1217) boils the edestin (2 grm.) with a solution of 75 grm. of tartaric acid and 10.5 grm. of Rochelle salt in 500 c.c. of water, adds 10 c.c. of a 1% solution of mercuric chloride and then 400 c.c. of water. This solution, which keeps well, is digested with 1 c.c. of pepsin in a test-tube at 40° for 30 minutes. The action is stopped by immersion in boiling water for 5 minutes,

and the solution titrated with a 20% solution of sodium chloride till the formation of a precipitate denotes the end-point. To obtain the edestin, Brewster (*J. Biol. Chem.*, 1921, **46**, 119) extracts de-fatted hempseed with a 5% solution of sodium chloride, recrystallises the extract, washes it with alcoholic solutions of increasing strengths and dries it with ether. According to Gross (*Annalen*, 1908, **33**, 130) caseinogen (1 grm. in 16 c.c. of 25% hydrochloric acid of sp. gr. 1.124, made up to 1,000 c.c.) is preferable to edestin, the undigested portion being precipitated with a 20% solution of sodium acetate.

Jacoby (*Biochem. Z.*, 1906, **1**, 53; 1908, **10**, 229) dissolves 0.5 grm. of ricin in 5% sodium chloride solution and filters. The opalescent solution becomes cloudy on the addition of 0.2*N* hydrochloric acid, and this solution is mixed with varying quantities of gastric juice and placed in a thermostat (37°) for 3 hours. The solution which has become clear is taken as that containing the minimum amount of enzyme able to digest the protein.

An elaborated form of Jacoby's method is suggested tentatively by the A. O. A. C. (*loc. cit.*, see p. 158). A fresh solution of ground and dried U. S. P. pepsin in 0.1*N* hydrochloric acid is prepared so as to give a strength of 0.5 mg. of "100% pepsin" per 100 c.c. Dried commercial ricin, passed through a No. 60 sieve, (1 grm.) is digested with 100 c.c. of 5% sodium chloride solution for 1 hour at 37.5°, filtered, and used at once. The acidity of the sample is adjusted by the addition of 0.2*N* hydrochloric acid or of water, so that at 37.5°, 1 c.c. takes approximately 15 minutes to digest 2 c.c. of ricin solution in the presence of 0.5 c.c. of 0.1*N* hydrochloric acid. To 50 c.c. of this enzyme solution are then added 40 c.c. of 0.1*N* hydrochloric acid, and a similar solution is inactivated by immersion in boiling water for 15 minutes, and filtered. Two c.c. of 0.1*N* hydrochloric acid are then added in each case. The activities of various volumes of the solution at 37.5° for 10 minutes may then be compared with those of known amounts of standard under the same conditions, in the presence of 2 c.c. of ricin solution. If the rate of digestion in the boiled control is at all appreciable, an interfering substance is present which may be removed by dialysis or evaporation under reduced pressure. The results are expressed as the percentage of "100% pepsin," 0.05 mg. of which completely dissolves the ricin

in less than 2 hours, whilst 0.005 mg. has a marked action in this time. Pepsin is absent if no effect is visible after 4 hours.

Mett's method is often used and is useful for dilute solutions. Fresh egg-albumin is drawn into a glass tube 1-2 mm. in diameter, coagulated at 95°, and the tube cut into 10 mm. lengths which are placed (in duplicate) in test-tubes for 10 hours at 37.5° with 1-2 c.c. of pepsin. The total length dissolved is then noted, and when this is about $\frac{3}{4}$ of the total, it is proportional to the amount of pepsin present. In the micro-method of Sastri and Sreenivasaya (*J. Ind. Inst. Sci.*, 1928, **11**, [A], 31) alternate layers of substrate mixed with boiled and unboiled enzyme solution are placed in capillary tubes which are sealed, warmed, and the increase in the size of the active layers observed under the microscope.

Titration Method, (Volhard and Löhlein).—Since casein combines with hydrochloric acid, the acid liberated during digestion of pepsin at 40° with a mixture of 150 c.c. of $N/14$ hydrochloric acid and 100 c.c. of a 5% solution of casein in N sodium hydroxide solution may be determined by titration before and after reaction. The undigested casein must be precipitated in each case by the addition of 100 c.c. of a 20% solution of sodium sulphate. According to the Schütz rule, pepsin concentration = $(\text{time} \times \text{amount of enzyme used})^2 / (\text{increase in acidity})^2$.

Physical Methods.—These give excellent results, but, as they require precision apparatus, are not practicable in cases where estimations are made only occasionally. The best is that of Northrup and Hussey (*J. Gen. Physiol.*, 1923, **5**, 353) who estimate under fixed conditions of time, temperature and pH, the change in the viscosity of a solution of pure gelatin under the influence of the enzyme. The time to effect a given change is inversely proportional to the amount of enzyme present. These authors also found that the percentage change in electrical conductivity during the peptic digestion of egg-albumin, which is constant for a given amount of pepsin, and independent of the original conductivity, provides an accurate means of measurement of the enzyme. At pH 2.6, which is produced by the addition of hydrochloric acid, the change is at a maximum. Rostock (*Chem. Zentr.*, 1924, **ii**, 2414) measures the increase in refractive index of coagulated blood fibrin, acted on by pepsin, by means of the immersion refractometer.

Valteich and Glover (Abs. *J. Chem. Soc.*, 1922, ii, 406) and Graber (*J. Ind. Eng. Chem.*, 1916, 8, 911) have compared some of the above methods. The methods of the U. S. P. and of Northrup are recommended, but storage eggs should be used in the former case.

TRYPSIN

This enzyme is present in the pancreas and is extracted by glycerin. Its inactive form (trypsinogen) is activated by enterokinase, from which it is separated by ultra-filtration (*vide infra*), and purified by re-precipitation with acetic acid from a solution in 0.03% ammonia. It breaks down proteins to polypeptides and amino-acids, and it is active in alkaline, slightly active in neutral, and inactive in acid solutions. It is very unstable in aqueous solution, but is protected by the presence of the substrate and of the products of hydrolysis. The optimum temperature is 40°, and the optimum pH for casein digestion 6.8–8.0, (Smorodinzev and Adova, *Z. physiol. Chem.*, 1926, 160, 189; Abderhalden and others, *Fermentforsch.*, 1929, 10, 474 *et seq.*).

Qualitative Tests.—These, in general, are similar to those described for pepsin, but reaction takes place only in solutions containing 0.2–0.5% of alkali. Thus, acid fibrin swells, but does not dissolve, whilst the production of tyrosine is shown by Millon's reagent. Trypsin gives the biuret, xanthoprotein, and Molisch tests.

Quantitative Methods.—Reference may also be made to pepsin in this case. The Fuld-Gross method is very suitable, particularly if Kai's modification is used (*J. Biol. Chem.*, 1922, 52, 133). Hammarsten's casein (0.1 grm.) is dissolved in 15 c.c. of a 0.1*N* solution of sodium hydroxide, neutralised to phenolphthalein, and diluted to 500 c.c. The sample (1 c.c.) is mixed with 20 c.c. of this solution at 40°, and at 5 minute intervals 2 c.c. are removed, and 1 c.c. of an aqueous solution of 17.2 c.c. of *N* sodium hydroxide solution, and 33.7 c.c. of *N* acetic acid in 100 c.c. added. No white precipitate is produced at the end-point, which should occur after 15–20 minutes for a standard (0.1%) trypsin solution. The time of digestion is inversely proportional to the amount of enzyme. Mett's method may be used in the presence of equal volumes of trypsin and a 0.4% solution of sodium bicarbonate; also that of Volhard and Löhlein, provided that the acid is added after, instead of before the digestion. A modification of the latter due to Willstätter and Persiel (*Z.*

physiol. Chem., 1925, **142**, 245) is very accurate, but is upset by inhibiting substances. The trypsin (10 mg.) is activated by enterokinase (see p. 178) at 37° for 30 minutes, and pH 8.0 obtained by the addition to 5 c.c. of 2 c.c. of a mixture of ammonium chloride and *N* ammonia (2:1). The mixture is maintained at 37° for 20 minutes with 5 c.c. of a 15% solution of gelatin, and then poured into 55 c.c. of boiling absolute alcohol, and titrated with a 0.2*N* alcoholic solution of potassium hydroxide till a grey to blue-green colour is produced with thymol-phthalein. The unit is an increase in acidity equivalent to 2 c.c. of alkali.

Jacoby (*Biochem. Z.*, 1908, **10**, 229) takes 1 grm. of ricin in 100 c.c. of 1.5% sodium chloride solution and adds to a series of tubes, each containing 2 c.c. of this solution, increasing quantities of a 1% trypsin solution. Each tube is made up to 3 c.c. with water, and 0.5 c.c. of a 1% sodium hydroxide solution added. All the tubes are opaque, but those which contain trypsin gradually become clear when maintained at 37°.

Van Slyke's method (see *Index*) may be used for the determination of the amino-nitrogen before and after hydrolysis.

Linderström-Lang (*Compt. rend. Trav. Lab. Carlsberg*, 1927, **17**, No. 4) has suggested for this determination, an improved method which is not influenced by the buffer solutions usually employed in enzyme reactions. The titration is carried out to match two colours, produced in controls (10 c.c. of water, 200 c.c. of pure acetone, and 10 drops of a 0.1% alcoholic solution of naphthyl red) by the addition of 0.53 and 1.10 c.c. of 0.1*N* hydrochloric acid (orange and red at pH 5.1 and 4.8, respectively). The acetone is added to 10 c.c. of the actual solution at the first stage, and the second is the end-point. Weakly basic and other exceptional nitrogen-containing groups (*e. g.*, in arginine, urea, creatine, guanidine, taurine, histidine, etc.) are not completely titrated.

Sörensen's Method (*Biochem. Z.*, 1907, **7**, 45).—This is based on the fact that carboxyl groups can be titrated with barium hydroxide in presence of amino groups if the latter are fixed in the form of methylene compounds by an excess of formaldehyde. The formaldehyde solution is freshly prepared from 50 c.c. of 40% formaldehyde, 25 c.c. of absolute alcohol and 1 c.c. of a 0.5% solution of thymolphthalein in 93% alcohol, and neutralised with a 0.2*N* solution of sodium or barium hydroxide. For the control

titration, 200 c.c. of boiled water, 15 c.c. of the formaldehyde mixture, and 5 c.c. of the barium hydroxide are back-titrated with 0.2*N* hydrochloric acid till the mixture has a bluish opalescence. Two drops of barium hydroxide now produce a blue coloration and a further two drops a stronger blue, which is taken as the end-point. A mixture of the hydrolysed protein solution (200 c.c.), 15 c.c. of formaldehyde, and a slight excess of barium hydroxide is titrated with hydrochloric acid till the colour is weaker than that of the control. Barium hydroxide is then added, and the colour of the control is attained. The number of c.c. of barium hydroxide used (less the blank), multiplied by 2.8, gives the amount of hydrolysed nitrogen in mg. With phenolphthalein the end-point is a distinct red colour.

A simpler method is to neutralise the formaldehyde with the alkali till it is just red to phenolphthalein. Two c.c. are added to the test solution, which is titrated until a red colour is obtained, which matches a control made by the addition of a known excess of alkali to a little phenolphthalein. Sørensen, Henriques and Gjaldbaek (*Z. physiol. Chem.*, 1911, **75**, 363) carry out the titration in four stages. The test liquid is made neutral to litmus paper and phenolphthalein. It is then titrated to a faint rose (first stage), next to a distinct red to match the control (second stage); formaldehyde is then added and titration continued to a faint rose (third stage), and finally to a distinct red (fourth stage). When the ratio of the figures in the fourth and first stages is high, amino-acids are indicated; when low, the products are of a polypeptide nature. For gastric juices, the modification of Leuders and Bergeim (*Amer. J. Physiol.*, 1923, **66**, 297) has been found simple, accurate and rapid.

If litmus is used as indicator, carbonic and phosphoric acids must first be removed by precipitation with barium chloride and hydroxide. A slight colour in the solution may be matched in the blank before the titration, but, when this is not possible, it may usually be removed by the addition to 25 c.c. of liquid of 4 c.c. of a 2*N* solution of barium chloride and 20 c.c. of a 0.3*N* solution of silver nitrate. The solution is made up to 50 c.c., shaken, and filtered.

Physical Methods.—Since the products of tryptic action have greater electrical conductivities than their substrates, the increase in value of this property may be measured (Bayliss, *J. Physiol.*,

1907, 36, 221). If optically active polypeptides are employed, an excellent indication of the progress of reaction is provided by the change in polarisation. Unchanged proteins may be removed by precipitation. The change in refractive index has also been measured.

Colorimetric Method. (Goldstein, *Fermentforsch.*, 1928, 9, 322).—A mixture of 2.5 gm. of casein, 20 c.c. of water and 16 c.c. of 0.1*N* sodium hydroxide solution is warmed to 50°, 15 c.c. brought to pH 7.7 by the addition of 2 c.c. of a mixture of *N*/3 mono- and disodium hydrogen phosphates in the proportions 1:9 by volume, and 2 c.c. of pepsin solution added. To each of 10 test-tubes are added 1.5 c.c. of this mixture, and the whole maintained at 38°, ten drops of 3% acetic acid and 1 drop of 0.5% bromine water being added to a tube every 5 minutes. The production of tryptophane is shown by a pink-violet colour, and it is claimed that this affords a more reliable indication of the course of the reaction than a determination of the final end-point. Unit activity is that of a solution which gives a coloration after 100 minutes.

Sherman and Newn (*J. Amer. Chem. Soc.*, 1916, 38, 2199) have compared the methods usually employed, and consider that the most accurate results are obtained by Van Slyke's method. The nitrogen digested is proportional to the amount of enzyme for 20–25 mg. and 40–60 mg. of nitrogen for pepsin and trypsin, respectively. Determinations of the total nitrogen in the digestion products are also useful, but Mett's method (p. 173) is reliable only for certain concentrations of enzyme. The optical method is delicate, but of use only for comparative purposes where large amounts of enzyme are concerned.

Anti-trypsin.—This enzyme, which occurs in blood serum, opposes the action of trypsin. Hence it may be measured or detected from the modifications produced by varying quantities in the measurement of known amounts of a standard trypsin by the methods described above (*e. g.*, Fuld's or Grützner's method). Achalme and Stévenin (*Compt. rend. Soc. Biol.*, 1911, 71, 333, 480) add varying quantities of anti-trypsin, a known amount of a standard trypsin, and a drop of mustard essence to 3 c.c. portions of milk, sterilised at 120°. The mixture which contains least anti-trypsin and is not coagulated after 20 hours at 50° gives the end-point. A standard trypsin, 0.01 c.c. of which should be neutralised

by 0.01 c.c. of anti-tryptic rabbit serum, is prepared from a macerated mixture of pancreatin and physiological salt solution kept at 35° for 20 hours. The filtered solution is kept in the dark with a drop of mustard oil.

Enterokinase is the autocatalytic, activating agent of the inactive pancreatic trypsin (trypsinogen), and occurs in the intestinal epithelium. It is extracted by water from the mucous membrane of the upper portion of the small intestine, and is separated from trypsin by ultra-filtration through a 4% acetic acid and collodion membrane. About 85% of the trypsin passes through and may be re-activated by addition to the residue (Bechold and Keiner, *Biochem. Z.*, 1927, 189, 1). Enterokinase is destroyed at 50°.

Trypsinogen may be extracted from the pancreas (with the intestine removed) at room temperature with 0.5% hydrochloric acid. The extract, which is strained, neutralised with sodium carbonate and kept under toluene, is activated by addition of the supposed enterokinase, and modifications of the tests described for trypsin applied. Thus, Mett's tubes may be made from gelatin coloured with methylene blue or gentian violet, placed in a mixture of 5 c.c. of a 0.5% solution of sodium carbonate with 2 c.c. of either (a) the enzyme mixture, (b) trypsinogen alone, (c) enterokinase and boiled trypsin, respectively. After 8-10 hours at room temperature (gelatin melts at 40°) the tube (a) only, should show a reaction. Centrifuged and sterilised milk also, may be clarified by the enzyme mixture.

Pancreatin has both diastatic and peptonising properties, though doubt has been thrown on the usual assumption that it is a mixture of diastase and trypsin by Rakuzin and Pekarskaja (*Abs. J. Chem. Soc.*, 1917, i, 422), who state that it does not give certain colour reactions associated with these enzymes.

The *Liquor Pancreatis* of the British Pharmacopœia (1914, p. 228) is prepared by the maceration of 250 grm. of fresh pigs' pancreas (free from fat or external membrane) for 7 days with 250 c.c. of 90% alcohol, 200 c.c. of glycerin, and 550 c.c. of water. The mixture is then triturated with fine sand or powdered pumice, and filtered. For the official test, 3 c.c. of solution, 0.2 grm. of sodium bicarbonate, 20 c.c. of water and 80 c.c. of milk are maintained at 40° for 1 hour, after which 5 c.c. placed in a test-tube with 5 c.c. of ether and 5

drops of nitric acid, and inverted 3 times, should produce no curdy precipitate in the bottom layer.

The pancreatin of the U. S. Pharmacopœia X (1925) converts not less than 25 times its weight of starch or of casein into soluble carbohydrates or proteoses, respectively. More active preparations may be diluted with lactose. The tests take account of both the peptonising and diastatic powers.—(a) *Starch Digestive Power*:—A weight of potato starch of known moisture content (which has been twice washed with 10 times its weight of cold distilled water, filtered, partly dried at 50°, powdered and maintained at 120° for 4 hours) equivalent to 7.5 gm. of the dry product, is dissolved in 200 c.c. of water and mixed with a solution of 0.3 gm. of pancreatin in 10 c.c. of water at 40° for 5 minutes. On the immediate addition of 0.1 c.c. to 0.2 c.c. of a 0.1*N* solution of iodine and 60 c.c. of water, no blue or red colour should be produced. (b) *Casein Digestive Power*:—A solution of 0.1 gm. of powdered casein in 30 c.c. of water and 1 c.c. of a 0.1*N* solution of sodium hydroxide is prepared at 40°, and made up to 50 c.c. To 5 c.c. are added 2 c.c. of a 0.02% aqueous solution of pancreatin and 3 c.c. of water, followed, after 1 hour at 40°, by 3 drops of a mixture of 1 c.c. of glacial acetic acid with 9 c.c. of water and 10 c.c. of alcohol. No precipitate should be produced.

Erepsin is obtained from the mucous membrane and from brewer's yeast. In the latter case the washed and pressed yeast is ground with sand, 30 gm. of calcium carbonate and 30 c.c. of chloroform, and after autolysis for 3 days at 20°, is filtered and dialysed in the presence of toluene. It breaks down albumoses, peptones and polypeptides into amino-acids, but, with the exception of casein, does not attack proteins. It is active only in an alkaline medium, and the optimum reaction is at 47–52° and *pH* 7.8.

Erepsin is measured from the change in electrical conductivity of the enzyme-substrate mixture, from the increase in amino-nitrogen produced during the reaction or from the rate of destruction of peptone. The substrate is Witte's peptone, 500 c.c. of which are digested with 100 c.c., or suitable varying quantities, of erepsin solution and 6 c.c. of toluene for 2 days at 37°. The decrease in the nitrogen content of the precipitate produced with phosphotungstic acid is an indication of the end-point, and the amounts of precipitable nitrogen which remain are 55, 36 and 21%

for pepsin, trypsin and erepsin, respectively. The digested solution should not give a marked biuret reaction (which, however, should be given by a control in which the enzyme solution has been boiled).

Papain is a proteolytic enzyme which occurs in the leaves, juices, fruits and milk of many plants (particularly *Carica*). The plant is ground in a ball-mill and extracted with equal volumes of water and glycerin. The enzyme reacts best at 65–70° and pH 5.0, and is assisted by the presence of hydrogen sulphide and hydrogen cyanide. It is measured by Fuld's method (p. 171).

AMIDASES

Arginase is found in the aqueous extract of mammal livers. Its presence is shown by the production of ornithine and urea from arginine sulphate, or from the large increases in formaldehyde-titratable nitrogen (see p. 175) it produces in a substrate made by the acid hydrolysis of edestin, (Clementi, *Atti R. Accad. Lincei*, 1915, **24**, i, 483; 1922, **31**, ii, 454). Poller (*Ber.*, 1927, **59**, [B], 1926) follows its action from the wine-red colour obtained with an alkaline solution of arginine in the presence of a few drops each of a 0.1% solution of α -naphthol in 70% alcohol, and of sodium hypochlorite (5%).

Clementi's method (*loc. cit.*) is also used for the quantitative measurement of arginine. The mixture of arginine sulphate (0.5 c.c. of a solution containing 0.8% of base) and enzyme is titrated before and after the reaction with 0.2*N*. sodium hydroxide solution in the presence of formaldehyde. The urea, arginine sulphate and ornithine sulphate react neutral, and as mono- and di-basic acids, respectively. Hunter and Dauphinee (*J. Physiol.*, 1924, **59**, 34) determine the urea produced in a given time colorimetrically from the change in pH value produced during decomposition of the urea by urease (*vide infra*) in the presence of a phosphate buffer of pH 6.9. At 50° and pH 7.0 arginase gives 92% of the theoretical yield of urea.

Asparaginase is of interest, since it removes the amide nitrogen of asparagine quantitatively, without affecting the nitrogen of the NH₂ group. Geddes and Hunter (*J. Biol. Chem.*, 1928, **77**, 197) obtained it from an aqueous extract of yeast cells or calves' liver. Its pH range is 5.5–10.3 (maximum 8.0), and it is destroyed by heat,

alcohol, or acetone. It is adsorbed on kieselguhr or on ferric hydroxide and precipitated by safranine at pH 4.5.

Urease is obtained from powdered soya bean by extraction with 0.01*N* hydrochloric acid. If to 250 c.c. of the filtered extract 5 c.c. of an aqueous solution of 70 gm. of disodium phosphate and 27 gm. of mono-potassium phosphate in 100 c.c. are added, it may be kept at -2° for 4 weeks without substantial loss of activity or increase in ammonia content. Sodium fluoride, which is often used to preserve blood, is injurious to urease, and if it is present the solution should be diluted, or the fluorides removed by precipitation as magnesium fluoride (Jacoby, *Biochem. Z.*, 1928, **198**, 163). The activity is destroyed if the extract is heated to 37° (Wester, *Chem. Weekblad*, 1919, **16**, 1461 and Groll, *id.*, 1527). Sumner (*J. Biol. Chem.*, 1926, **69**, 435; **70**, 97) extracts the meal with 5 parts of 32% acetone for 12 hours at 2° , and obtains a crystalline product which may be kept in the dry state.

The production of ammonia from urea is used as a means of detection and estimation both of urease and of urea. The original solution may be freed from ammonia by means of powdered permutite (0.2 gm. per c.c.). Fiske (*J. Biol. Chem.*, 1915, **23**, 453) adds carefully to a mixture of urea, urease and a drop of kerosene in a glass tube fitted with a stopper and an inlet tube, 5 c.c. of a filtered solution of 500 gm. of potassium carbonate with 10 c.c. of a 30% solution of potassium oxalate in 500 c.c. of water. The ammonia may then be determined with an accuracy of 0.05% by drawing ammonia-free air through the solution for an hour, and absorbing it in 0.02*N* hydrochloric acid, the excess of which is subsequently titrated with 0.01*N* sodium hydroxide solution, with methyl red as indicator. It is advisable to remove phosphates by precipitation with baryta, the excess of which is precipitated (with any calcium) by sodium carbonate. Sumner (*loc. cit.*) measures the quantity of urease to produce 100 mg. of ammoniacal nitrogen after 5 minutes at 20° , whilst Pincussen (*Biochem. Z.*, 1922, **132**, 242) describes a micro-method in which a micro-Kjeldahl determination of the non-protein nitrogen formed during the reaction is made after removal of the unchanged protein by colloidal iron. The optimum pH of the reaction is 7.0 (Van Slyke and Zacharias, *J. Biol. Chem.*, 1914, **19**, 181).

Oxidising and Reducing Enzymes

Oxydases.—Enzymes are supposed to take part in a large variety of oxidation changes undergone by different materials, such as sugars, fats, amino-acids, aldehydes, alcohols, phenols, etc. Of the reactions proposed for their detection, none is really satisfactory nor are they given by all oxydases, and often the test is given slowly when no oxydase is present. Very considerable experience is therefore necessary in working with them, and a wholly satisfactory test is greatly to be desired. The difficulty is, perhaps, in part due to the peculiar nature of oxydases. Oxydases react at pH 7–10.

Oxydase¹ is readily extracted with water from the potato tuber, from maize, and from fungi, particularly the *Lactarius* species. The tests for oxydases are nearly all based on colour reactions, and the chief reagents are given below, with references to the original papers. Usually a few drops of the reagent are added to 2 or 3 c.c. of the suspected enzyme solution. An immediate colour indicates oxydase, but if it appears only after the addition of a few drops of hydrogen peroxide, the presence of peroxydase is indicated. The technique for the macro- and microscopic identification of oxydases in the flowers and vegetative parts of plants by means of benzidine and α -naphthol has been described by Keeble and Armstrong (*Proc. Roy. Soc.*, 1912, [B], 85, 214).

(1) Guaiaconic acid or guaiacum, in the form of a 1% tincture in alcohol, freshly prepared or freed from peroxides by boiling with animal charcoal, turns blue with oxydase (Moore and Whitely, *Biochem. J.*, 1909, 4, 136).

(2) A 1% solution of α -naphthol in 50% ethyl alcohol gives a lavender colour when oxidised, and is suitable for microscopic work (Bourquelot, *Compt. rend.*, 1896, 123, 423).

(3) *p*-Phenylenediamine hydrochloride. A 1% solution in water, yields a greenish colour.

(4) Indophenol. Three drops of a mixture of reagents (2) and (3) are added to the enzyme extract, which is then made slightly

¹ It is desirable to use the general term "oxydase" rather than the more specific "laccase," as it is improbable that there are specific oxydases for each class of compounds. The term "laccase" (or phenolase) has also been applied specifically to enzymes which oxidise aromatic amines, or phenols, with the production of a pigment (*e. g.*, tyrosinase). Pugh and Raper (*Biochem. J.*, 1927, 21, 1370), however, consider a division of oxydases in aerobic and anaerobic types to be justified.

alkaline with sodium carbonate. The purple oxidation product then dissolves. Vernon (*J. Physiol.*, 1911, **42**, 402) and Laskowski, (*Compt. rend. Soc. Biol.*, 1928, **98**, 1369) have adapted this test for quantitative work.

(5) A pinch of phenolphthalein is dissolved in 1 c.c. of 0.1*N* sodium hydroxide solution, 25 c.c. of water are added, and the solution filtered and made up to 100 c.c. Five c.c. are left for 18 minutes with the oxydase, when the mixture acquires a red colour due to phenolphthalein which may be matched against a standard (Kastle, *Amer. Chem. J.*, 1908, **40**, 251).

(6) Phenol. A 5% aqueous solution becomes reddish-brown in 24 hours (Bourquelot, *Compt. rend.*, 1896, **123**, 315).

(7) A 2% alcoholic solution of guaiacol, which is more stable than the guaiacum tincture, is oxidised to the red tetraguaiacoquinone $[C_6H_3(OCH_3)O]_4$ (Bourquelot, *Compt. rend. Soc. Biol.*, 1896, **48**, 893). Fleury has made this reaction quantitative (*vide infra*).

(8) Quinol is oxidised to quinone (Bertrand, *Compt. rend.*, 1894, **118**, 1215).

(9) Benzidine. (Schreiner, *U. S. Dept. Agric., Bureau of Soils, Bull.*, 36, 1909.) A 1% solution in 50% ethyl alcohol gives an intense blue coloration which changes to a brown precipitate.

(10) Salicylic aldehyde is oxidised to salicylic acid (Schmiedeberg, *Arch. exp. Path.*, 1881, **14**, 288, 379; Jaquet, *id.*, 1892, **29**, 386; also Jacoby, *Z. physiol. Chem.*, 1900, **30**, 135).

Oxydase has been determined quantitatively by the following methods:

(a) Oxidation of potassium iodide and acetic acid, the iodine liberated being measured with a solution of sodium thiosulphate (Bach, *Ber.*, 1904, **37**, 3785).

(b) Oxidation of pyrogallol to insoluble purpurogallin (Bach and Chodat, *Ber.*, 1903, **36**, 1756; 1904, **37**, 1342).

(c) Oxidation of the leuco-base of malachite green to the blue-green pigment (Czyhlarz and von Fürth, *Beitr. chem. physiol. Path.*, 1907, **10**, 358).

(d) Oxidation of vanillin to dehydrovanillin (Herzog and Meier, *Z. physiol. Chem.*, 1908, **57**, 35; 1909, **59**, 57; 1911, **73**, 258), in which 150 c.c. of a 0.96% solution of vanillin, 5 c.c. of hydrogen peroxide (10 volumes), and the enzyme solution are made up to 400 c.c. and allowed to stand for a convenient period of 12 to 24 hours. The

precipitate of dehydrovanillin is collected on a tared filter, washed, dried at 100° and weighed. This method has been found to give satisfactory results for the comparison of the activities of several enzyme solutions, but it is in no way an absolute measure of the activity.

(e) Oxidation of the leuco-base of brilliant green (Herzog and Polotzky, *Z. physiol. Chem.*, 1911, 73, 247).

(f) Fleury (*J. Pharm. Chim.*, 1924, 29, 402) aspirates air through a mixture of guaiacol and the enzyme (see above), extracts the coloured product with chloroform, and matches it colorimetrically. The colour due to 46.5 mg. of tetraguaiacoquinone extracted from an aqueous solution in 1 litre of chloroform is the same as that of a 0.01*N* solution of iodine, when both are viewed through 10 mm. of solution. The oxidation reaction is quantitative so long as (i) it is not unduly prolonged, (ii), there is no excess of guaiacol or alkali, and (iii) the temperature is low. The limitations of the reaction as applied to plant extracts are discussed by Onslow (*Biochem. Z.*, 1924, 18, 549).

(g) Goldenburg compares the oxidation effects of oxydase with that of a 0.01*N* solution of potassium persulphate and measures the amount required to cause reduced eosin¹ to absorb 1 c.c. of this solution. If the solution is turbid, the regenerated eosin is precipitated with 2% hydrochloric acid, filtered, washed with the acid, redissolved in a 2% solution of sodium hydroxide and the colour matched. Traces of copper also regenerate the eosin and upset the reaction.

Harvey (*J. Gen. Physiol.*, 1920, 2, 253) has suggested an improved form of Bunzel's method (*J. Biol. Chem.*, 1914, 17, 409), in which the volume change due to absorption of oxygen during reaction in a closed vessel, is measured by a gauge. Carbon dioxide is absorbed by sodium hydroxide, and the apparatus may also be used for measurements of catalase.

Uricase is an oxydase extracted from wheat or hashed frogs' tissue by digestion with chloroform, glycerin and water for 2 days at 20° . It contains sulphur, phosphorus and traces of nitrogen, degrades uric acid (cf. urease p. 181), and gives faint biuret, xanthoprotein and Millon reactions but a negative tryptophane test.

¹ The eosin (1 grm.) is reduced by 5 grm. of zinc and 2 grm. of sodium hydroxide in 100 c.c. of re-distilled water, and the solution diluted 200 times.

It reduces Fehling's solution and is activated by copper but destroyed by fluorides, cyanides, alcohol and mercuric chloride (Przylecki, *Compt. rend. Soc. Biol.*, 1928, **98**, 787 *et seq.*).

Plant Oxydases.—In such cases it is probable that the best results will be obtained by the use of several oxydase reagents, in order to find that most suited to the particular case. One of the most satisfactory reagents is benzidine, used either as a 0.5% solution in 50% ethyl alcohol or as a saturated solution in 1 or 2% sodium chloride solution (Keeble and Armstrong, *Proc. Roy. Soc.*, 1913, **87**, [B], 125). Blue or violet-brown colorations or precipitates are obtained when the reaction is positive, owing to the formation of meri-quinonoid salts of diphen-quinonediimine. Chodat (*Arch. Sci. phys. nat.*, 1912, **33**, [iv], 70) has shown that a remarkable range of coloured compounds is produced when a vegetable oxydase acts on *p*-cresol in the presence of an amino-acid, polypeptide or peptone, according to the nature of the amino-compound. Kober (*J. Amer. Chem. Soc.*, 1914, **36**, 1304) applies the estimation of suspensoids by means of the nephelometer to the study of enzymes. In the case of the nucleases, undigested nucleic acids are precipitated by a 0.2% solution of egg albumin faintly acidified with acetic acid, and are matched. Bunzel's method (see p. 184) is also used.

Peroxydase is contained in the aqueous extracts of most plants. Willstätter and Stoll (*Annalen*, 1918, **416**, 21) extract the crushed plant with oxalic acid, and make the extract alkaline with baryta, the excess of which is precipitated by carbon dioxide and filtered off. The enzyme is precipitated from the concentrated filtrate with alcohol.

Peroxydase is active only in the presence of hydrogen peroxide, when it behaves in the same way as oxydase. The above tests may then be applied.

The guaiacum test is used to distinguish between boiled and unboiled milk. Fresh milk gives a blue coloration with guaiacum tincture and peroxide, but boiled milk gives no reaction. With 5 drops of *p*-phenylenediamine (2%) and a drop or two of dilute peroxide (1 in 10), fresh milk (5 c.c.) gives a yellow colour which changes rapidly to emerald green and Prussian blue, whilst boiled milk gives the reaction only after several hours. With concentrated hydrogen peroxide the boiled milk gives a blue colour immediately

(Nicholas, *Bull. Soc. Chim.*, 1911, 9, 266-269). Such methods must be used with discrimination, as a colour may also be produced by foreign organic substances (see Violle, *Compt. rend.*, 1919, 169, 248). Hinks (*Analyst*, 1915, 40, 483) finds that whilst *p*-phenylenediamine is the best reagent for general purposes, benzidine is very sensitive under certain conditions. The peroxide should be floated in a layer on to the milk. Bach (*J. Chem. Soc.*, 1916, i, 682) uses *o*-cresol or saligenin, which are oxidised to a yellow compound which then turns red-brown. Holborow (*Analyst*, 1929, 658) uses ortol.

Bansi and Ucko (*Z. physiol. Chem.*, 1926, 157, 192), as a result of comparative tests of the above methods, recommend the cautious use of guaiacol in preference to pyrogallol or cresol, since the resulting colours are more stable and more comparable. Impurities such as iron, however, may accelerate the action. The use of ethyl hydrogen peroxide, which is more stable than hydrogen peroxide and gives a brick-red colour in the presence of guaiacol, is advocated by Grimmer (*Milchw. Zentr.*, 1915, 44, 246) for milk tests, but is also not without objection. Gramenitzki (*Biochem. Z.*, 1927, 185, 433) considers the guaiacum test unsuitable for blood in the presence of a relatively small amount of hydrogen peroxide, since it is masked by the catalase reaction (*vide infra*).

For the quantitative estimation Bach and Zoubkoff (*Compt. rend.*, 1920, 170, 967) match the colour produced from 1 c.c. of a 1% solution of guaiacol with 1 c.c. of enzyme solution and 7 c.c. of water, with that of a boiled and filtered solution of 5 grm. of egg albumin, 2 grm. of cobaltous chloride, and 10 grm. of sodium hydroxide in 250 c.c. of water. The solution is standardised against known amounts of guaiacol oxidised as described above.

Rice and Hanzawa (*J. Ind. Eng. Chem.*, 1922, 14, 201) and Willstätter and Pollinger (*Z. physiol. Chem.*, 1923, 130, 281) have applied Bach and Chodat's method to milk and to plant products. The former authors centrifuge the warm milk to remove the fat, and place 10 c.c. in a stoppered bottle with 20 c.c. of a 5% solution of pyrogallol, 20 c.c. of 1% hydrogen peroxide, and 110 c.c. of water for 7 days at room temperature. The precipitated purpurogallin is filtered off on a weighed Gooch crucible, washed with water till the washings no longer give a dark blue colour with ferric chloride, freed from fat with petroleum spirit, dried and weighed. The peroxylase number is the weight of precipitate. This method,

also, is useless in the presence of oxidising agents, catalase, mercuric chloride (which precipitates casein) or formaldehyde (which reduces the peroxide), (Kirchner and Nagell, *Biochem. Z.*, 1926, **174**, 167).

In preference to pyrogallol methods, however, Willstätter and Weber (*Annalen*, 1926, **449**, 156) recommend the simple oxidation of the leuco-base to malachite green in the presence of dilute acetic acid and sodium acetate solutions, by hydrogen peroxide, under the influence of peroxidase. The colour produced in 5 minutes is matched colorimetrically, and in the absence of a high concentration of salts, too low a concentration of enzyme, or an excess of peroxide, the error is only 1.5%.

Tyrosinase (Phenolase or laccase¹) also occurs in many plant extracts and particularly in fungi (*Russula* and *Agaricus* species) and wheat bran. The filtered extract from the maceration of the material with chloroform-water is precipitated with alcohol, and the enzyme washed with alcohol and dried *in vacuo*. Animal tyrosinase is obtained from the sac of the cuttle fish.

Tyrosinase which acts only on tyrosine or allied compounds, and which is inactivated at 70°, is identified by the production of black insoluble melanin when it acts on a 0.05% solution of tyrosine in dilute sodium carbonate solution (0.04%), and by the black colour produced with quinine sulphate (Boas and Merkschlager, *Biochem. Z.*, 1925, **155**, 197). A red substance, which turns colourless and then black, is produced in the former reaction as an intermediate product, and though these last two stages of the reaction occur in the absence of the enzyme, they are accelerated by it. Raper and others (*Biochem. J.*, 1923, **17**, 454; 1927, **21**, 1370) consider the pH value, which should be about 8, to be an important factor. Haehn (*Biochem. Z.*, 1920, **105**, 169) has found that characteristic colours are obtained when this reaction takes place in the presence of neutral salts which activate the enzyme and precipitate the colloidal melanin. Zinc, cadmium and calcium salts may be used, but an excess of hydrogen, hydroxyl, acetic, lactic or halogen ions retards the action. Bach (*Ber.*, 1908, **41**, 216, 221) has shown that the amount of change in this reaction can be determined quantitatively by titration with potassium permanganate (0.002*N*) and sulphuric acid (10%). Haehn and Stern (*Biochem. Z.*, 1927, **184**, 182) precipitate the pigment by

¹ See footnote on p. 182

barium chloride from an alkaline solution, and determine the residual tyrosine in the clear filtrate. This is a rapid method. The spectroscopic measurement of melanin is very accurate for small amounts of enzyme. Other methods are the measurement of the volume of centrifuged sediment, or titration with 0.002*N* potassium permanganate solution till the dark colour disappears.

Schardinger's enzyme, (Aldehydase, salicylase, dehydrogenase, perhydridase), is intermediate in properties between an oxydase and reductase. Schardinger (*Chem. Ztg.*, 1908, **28**, 1113) noticed that an aldehyde added to fresh milk which was inert towards the methylene blue reaction, produced a positive reaction. Boiled milk gave no reaction. The aldehyde and substrate therefore act as oxygen and hydrogen acceptors, respectively. The enzyme, which is extracted from skim milk or butter by freshly distilled acetone, also reduces nitrates to nitrites (Sbarsky and Michlin, *Biochem. Z.*, 1925, **155**, 485).

It is measured by the colorimetric determination of the nitrites produced in a mixture of acetaldehyde, sodium nitrate and milk at 60° for 30 minutes in a closed vessel. If the proteins are precipitated by basic lead acetate solution and filtered off, the filtrate may be tested with α -naphthylamine (Bach, *Biochem. Z.*, 1911, **31**, 443).

Reductase (Hydrogenase) occurs in milk, and is detected by its power to reduce methylene blue to a colourless compound. In the method suggested by Barthel and Orla-Jensen (*Milch. Zentr.*, 1912, **14**) 40 c.c. of sample in a sterile tube (length 19 cm., diameter 2 cm.) are shaken with 1 c.c. of 0.20% methylene blue, and the time required to produce complete decolorisation at 38–39° is determined. Light, which accelerates the reaction, should be excluded. The effect of the colloidal substances in the milk (protein and fat) on this reaction has been studied by a number of workers. Eichwald (*Z. Unters. Nahr. Genussm.*, 1919, **38**, 359) considers that if the colloids are highly dispersed, the rate of decolorisation is slowed down, owing to adsorption of the unchanged indicator. Virtanen (*ibid.*, 1924, **48**, 141), on the other hand, found that dilution or the addition of salts and colloids produced no such effect. Arup (*Analyst*, 1918, **43**, 20) also discussed the precautions necessary and recommended a temperature of 28–29°. Soep (*Le Lait*, 1927, No. 70) prefers the use of 1 c.c. of a 0.01% solution of Janus green (safrana-

azo-dimethyl aniline) with 10 c.c. of milk. Unlike methylene blue, this dye is not back-oxidised by atmospheric oxygen. It changes from blue-green, through red and violet, to colourless.

Tankard however, (*Analyst*, 1928, 53, 213) points out that in any case oxidation is prevented by the rising of the cream, with the formation of a protective layer over the milk, and suggests that better differentiation may be obtained with Janus green by incubation at 42° instead of 37°.

Barthel and Jensen (*loc. cit.*) use the reductase test as a measure of the biological activity of the milk. Their scale, which follows, is useful if used with caution, since Dychno and Briskin (*loc. cit.*, p. 190) have shown that the time of decolorisation decreases as the acidity (particularly that due to lactic acid) increases, and also increases with the extent to which the milk has been previously heated. Tankard (*loc. cit.*) also obtained lower bacterial counts than those given.

Class	Description of milk	Time in hrs. for decolorisation		Number of bacteria per c.c. $\times 10^{-6}$
		Methylene blue	Janus green	
1	good	over 5½ (minimum)	over 6	under 0.5
2	indifferent	2-5½	3-6	0.5-4
3	bad	0.3-2	1-3	4-20
4	very bad	under 0.3	0-1	over 20

CATALASE AND ZYMASES

Catalase occurs principally in blood (*hæmase*), malt and tobacco leaves, and may be extracted with water. Its powers of decomposition of hydrogen peroxide are greatest at 2° and pH 6 (Burge, *Ann. J. Physiol.*, 1916, 41, 153), but it is inactivated by a temperature higher than 47° at a rate which depends on the pH value. A catalase unit is defined by Morgulis and Beber (*J. Biol. Chem.*, 1927, 72, 91; 1928, 77, 115) as that amount which will decompose 70% of the hydrogen peroxide present in the substrate.

For catalase in blood Bach and Zoubkoff (*Compt. rend.*, 1920, 171, 967) add 8 c.c. of a dilute solution to 2 c.c. of neutral 1% hydrogen

peroxide in a 75 c.c. flask, which is maintained at 37° for 30 minutes. Three c.c. of *N* sulphuric acid are then added, and the contents titrated with a 0.1*N* solution of potassium permanganate. The titration from a control, in which the enzyme solution is first boiled, is deducted. In a later paper (*Biochem. Z.*, 1921, **125**, 283) they suggest 17° as a temperature at which the catalase is less inactivated, and the disturbing influence of proteolytic enzymes consequently less marked than at 37°. Walling and Stoland (*ibid.*, 1923, **66**, 503) state that consistent and reliable results are obtained if the reaction proceeds at 22° at *pH* 8.9. On the other hand, the effects of anti-catalase are eliminated by the use of a lower temperature and a lower *pH*. Tsuchihashi (*Bioch. Z.*, 1923, **140**, 63) uses 90 c.c. of *N*/150 hydrogen peroxide with 10 c.c. of a phosphate buffer of *pH* 7.0. Such mixtures are kept at 2° with suitable quantities of enzyme, and the solution finally titrated in the presence of 20 c.c. of 20% sulphuric acid. A control is necessary. The iodine liberated from potassium iodide by the undecomposed peroxide may also be titrated.

The volume of oxygen evolved may also be measured. Van Laer (*J. Inst. Brew.*, 1909, **15**, 553) measured the gas 1 minute after mixing 6 grm. of malt and 25 c.c. of 0.85% hydrogen peroxide. Apparatus in which the solutions can be mixed and the volume of gas measured in a closed system have been described by Harvey (*loc. cit.* p. 184) and others. Thus, Machens and Cordes (*Milch. Zentr.*, 1921, **50**, 25) connect the shoulders of two similar bottles (one of which contains a vertical graduated tube filled with water to the zero-mark, and the other 15 c.c. of milk) by a short tube provided with a 3-way tap which can connect either or both with the air. The bottles are kept at 37° for 15 minutes in contact with air, 5 c.c. of 1% hydrogen peroxide then added to the milk, the bottles connected, and the oxygen evolution at 37° read from the motion of the water-level in the graduated tube. The Lobeck catalasometer (Dychno and Briskin, *Z. Unters. Lebensm.*, 1927, **54**, 438) is similar in action, but consists of a vertical cylinder, through the stopper of which a narrow graduated tube, open at both ends, passes and dips into the mixture.

Where direct titration is preferred, Charmandarian's method which is a measure of the degree of maturity of malt (*Biochem. Z.*, 1929, **204**, 389) is to be recommended. The ground and sieved sample

(1 grm.) is extracted for 1 hour with 50 c.c. of water, and 10 c.c. of the filtered extract maintained at 20° for 30 minutes with 10 c.c. of water and 2-5 c.c. of 1% hydrogen peroxide. The mixture is titrated with potassium permanganate solution in the presence of 5 c.c. of 10% sulphuric acid, and the blank titration obtained with catalase, inactivated after 5 minutes at 100°, deducted.

Catalase is activated by divalent cations (particularly Ca^{++}), such as are found in carbonated waters, but is inhibited by sulphur (particularly as sulphate). It is opposed by **Anticatalase**, an enzyme found in animal tissues, whose optimum reaction is at pH 6.7 and 38-40°.

Zymase produces carbon dioxide from 1% solutions of glucose, fructose, maltose or cane sugar, but not from lactose, and only slowly from galactose. The activity of yeast juice or acetone-yeast is estimated from the gas evolved. Twenty c.c. of yeast juice, 8 grm. of sucrose and a little toluene are mixed in an Erlenmeyer flask closed by a Meissl tube (see Vol. I, p. 290), and the loss in weight determined from day to day at 22°. This amounts to from 1-2 grm. When a more accurate measurement is required the carbon dioxide is replaced by air and the volume or weight measured, (compare Harden, Thompson and Young, *Biochem. J.*, 1910, 5, 230).

For the detection of minute quantities of alcohol (0.002% by volume) the so-called Pasteur drop method, adapted by Hansen and studied by Klocker (*Centr. Bakt.*, 1911, 31, 108), is of value. Five c.c. of the liquid are placed in a test-tube closed by a cork through which a glass tube, 80 cm. long, can pass. The whole is supported obliquely over a tiny flame and the liquid slowly warmed, when characteristic oily drops are clearly seen in the tube. The less alcohol present, the higher up the tube they appear.

Co-zymase is also obtained from yeast, and the intensity of the phloroglucinol pentose reaction is a measure of its activity. It is essential for the fermentation of sugar (Myrbäck, *Z. physiol. Chem.*, 1928, 177, 158, 237) and is probably a nucleotide. Oxidising agents do not affect it, but it is precipitated by silver nitrate and phosphotungstic acid from solutions in nitric and hydrochloric acid, respectively.

Carboxylase is analogous to zymase, and oxidises carboxyl groups, with the formation of carbon dioxide. It is detected or

measured by the formation of this gas and of acetaldehyde from a 0.1*N* solution of pyruvic acid.

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PUTREFACTION BASES

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Dead animal and vegetable tissues in nature undergo putrefactive decomposition by bacteria. The proteins are first hydrolysed to their constituent amino acids and then the latter are further broken down in one of two ways; most commonly they are desaminated and lose ammonia, so that no basic products are formed (except in the case of the diamino acids, where iminazolyl propionic, ϵ -amino caproic and ϑ -amino-valeric acids may arise, respectively, from histidine, lysine and arginine). Alternatively, the amino acids are decarboxylated, when bases result, some of which are interesting on account of their pharmacological action. Certain putrefaction bases superficially resemble the simpler vegetable alkaloids like coniine. Attention was first called to this by Selmi in 1870, after a medico-legal examination of human corpses. He suggested the name ptomaine (πτῶμα, corpse) for such bases, but did not obtain them pure. The first ptomaine to be analysed was a substance $C_8H_{11}N$ (Nencke, 1876) and was doubtless phenyl-ethyl amine (from phenyl-alanine by decarboxylation). Brieger began his extensive researches on ptomaines in 1883, and a number of his bases were certainly obtained in a state of purity. Many of the older and some more recent investigators have, however, described a large number of impure putrefaction bases which need not concern us here. The popular notion that "ptomaines" are the cause of food poisoning is erroneous. Even the more active are not particularly poisonous when taken by the mouth. Food poisoning is rather due to infection by a few specific bacteria, such as *Bacillus botulinus* and Gaertner's bacillus, which apparently synthesise complex and extremely poisonous toxins, quite different from the ptomaines, which are degradation products of amino acids. In order to avoid this confusion, the term "ptomaine" had better be abandoned.

The symptoms resulting from eating poisonous food usually begin with nausea, vomiting, pain in the abdominal region and diarrhoea, the faeces being frequently of an offensive character. Other symptoms are commonly faintness, muscular weakness, prostration and sometimes spasms, followed by fever, headache and thirst. Convulsions, clonic spasms, dilatation of the pupil and disturbance of vision, with drowsiness and occasionally coma, are also observed. The *post-mortem* appearances are similar to those produced by mineral irritant poisons. Whereas ordinary poisons generally show their effect within three to four hours after ingestion, the symptoms of food poisoning due to bacteria rarely become fully developed in less than six to eight hours, and in some cases are much longer delayed, probably because the micro-organisms have first to multiply and secrete the toxin.

Although animal tissues consist largely of proteins, small quantities of other nitrogenous substances are also present, and these too may yield putrefaction bases. Thus trimethylamine in herring brine is derived from choline, out of lecithin. Small quantities of creatine and other bases in muscle may, perhaps, also give rise to putrefaction bases.

The bases whose physiological actions have been investigated in detail are *iso*-amyl amine, phenyl-ethyl amine, indole-ethyl amine and particularly *p*-hydroxyphenyl-ethyl amine and iminazoly-ethyl amine (*q.v. infra.*).

CLASSIFICATION OF PUTREFACTION BASES

We first describe those bases which are derived from known amino acids; here we are on solid ground, for the constitutions have been established by synthesis and the chemical properties are fully known. Then follow a few bases of known constitution from other known or unknown sources, and finally a few bases of more doubtful character which have been imperfectly investigated. Many of the older observations, referring to bases of an entirely problematical nature, are not mentioned at all.¹

¹ For a more detailed account of the older literature consult L. Brieger, *Ueber Ptomaine*, Berlin, August Hirschwald, 1885-1886 (3 parts); for the technique of isolation the article by D. Ackermann, *Die Isolierung von Fäulnisbasen*, in Abderhalden's *Handbuch der biochemischen Arbeitsmethoden*, 1910, vol. II, pp. 1002-1043.

For an account of the modern literature see G. Barger, *The Simpler Natural Bases*, London, Longmans, Green & Co. 1914; M. Guggenheim, *Die biozenen Amine*, Berlin, Julius Springer, 1924; P. Hirsch, *Die Einwirkung von Mikroorganismen auf die Eiweisskörper*, Berlin, Gebrüder Borntraeger, 1918.

I. Putrefaction Bases, Certainly or Probably Derived from Amino-acids

A. Derivatives of Monamino-acids

Methylamine, CH_3NH_2 , has frequently been found, *e. g.*, in putrid fish by Bocklisch (*Ber.*, 1885, **18**, 1922). It is separated by steam distillation; its formation from glycine by loss of carbon dioxide is less probable than its formation from trimethylamine (*cf.* Ackermann and Schütze, *Arch. Hyg.*, 1910, **73**, 145).

Isobutylamine, $(\text{CH}_3)_2\text{CH}\cdot\text{CH}_2\cdot\text{NH}_2$, obtained by Neuberg and Karczag (*Biochem. Zeit.*, 1909, **18**, 434) from the putrefaction of *d-l-α-aminoisovaleric acid* (racemic valine). It is isolated as platinichloride after steam distillation and extraction of the distillate with ether.

Isoamylamine, $(\text{CH}_3)_2\text{CH}\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{NH}_2$, is derived from leucine by loss of carbon dioxide. For the conditions of this decarboxylation by a pure culture of *B. proteus* see Arai (*Biochem. Zeit.*, 1921, **122**, 251). It has been found in cod-liver oil (obtained by the old putrefaction process), in putrid yeast, in putrid horse meat (Barger and Walpole, *J. Physiol.*, 1909, **38**, 343) and in putrid placenta (Rosenheim, *J. Physiol.*, 1909, **38**, 337), also in Roquefort cheese (Nencki, *J. prakt. Chem.*, 1882, **26**, 47), in ergot (Barger and Dale, *J. Physiol.*, 1909, **38**, lxxvii) and in an edible fungus *Boletus edulis* (Reuter, *Z. physiol. Chem.*, 1912, **78**, 167). The isolation of this base is readily accomplished by utilising its volatility with steam and its solubility in chloroform and ether. By the addition of anhydrous oxalic acid to the dry ethereal solution, the *oxalate*,



separates out; this salt can be recrystallised from a mixture of acetone and alcohol and after drying in a vacuum, melts at 169° . It is slowly decomposed at 100° . On distillation with lime it yields the free base, which boils at 95° and is readily soluble in water. The *hydrochloride* is extremely soluble in water and therefore somewhat difficult to crystallise, but the *hydrobromide* readily separates out on adding alcoholic hydrogen bromide to an ethereal solution of the base. It forms glistening leaflets, *m. p.* 225° . 1 c.c. of an *N*/10 solution of an *isoamylamine* salt produces a marked rise of arterial blood pressure in the cat. For a detailed account of the physiological action,

(see Dale and Dixon, *J. Physiol.*, 1909, **39**, 25; compare also Barger and Dale, *J. Physiol.*, 1910, **41**, 25).

β -Phenylethylamine, $C_6H_5.CH_2.CH_2.NH_2$, was obtained by Nencki in the putrefaction of gelatin mixed with ox-pancreas. He and others described this base as collidine, without proving it to be a pyridine derivative. Spiro (*Beitr. z. chem. Physiol. Path.*, 1902, **1**, 347) proved its true nature and origin from phenylalanine. For details of this decarbonylation by *B. proteus* and *B. subtilis* see Amatsu and Tsudji (*Act. Schol. Med. Univ. Kyoto*, 1918, **2**, 447). Phenylethylamine has also been isolated from putrid horse meat (Barger and Walpole, *J. Physiol.*, 1909, **38**, 343). It is a liquid, boiling at 197° , which readily attracts atmospheric carbon dioxide to form a crystalline carbonate. The base is somewhat soluble in water, from which it may be extracted by ether or chloroform. The action of phenylethylamine on the blood-pressure is similar to that of *isoamylamine*, but more powerful. (Barger and Dale, *J. Physiol.*, 1910, **41**, 28.)

Tyramine, *p*-Hydroxyphenylethylamine, $OH.C_6H_4.CH_2.CH_2.NH_2$, is formed from tyrosine by putrefaction (Barger and Walpole, *J. Physiol.*, 1909, **38**, 343), and was first obtained from human corpses by Brieger, who called it "mydin" (*Ueber Ptomaine*, Pt. III, 1886, p. 26). Brieger already remarked on its innocuous character. It was then obtained in the autolysis of pancreas (Emerson, *Beitr. chem. Physiol. Path.*, 1902, **1**, 501) and in the prolonged peptic digestion of egg albumin (Langstein, *ibid.*, 1902, **1**, 507). In both these cases the digestion mixture was probably not sterile. The base was further found by Van Slyke and Hart in Cheddar cheese (*Amer. Chem. J.*, 1903, **30**, 8), by Gautier (*Bull. Soc. Chim.*, 1906, [iii], **35**, 1195) in putrid cod-livers, by Winterstein and Küng (*Z. physiol. Chem.*, 1909, **59**, 138) in Emmentaler cheese, by Barger and Walpole (*J. Physiol.*, 1909, **38**, 343) in putrid horse meat, by Rosenheim (*ibid.*, 1909, **38**, 337) in putrid placenta, and by Barger (*J. Chem. Soc.*, 1909, **95**, 1123) in ergot. For the preparation from tyrosine in a 70% yield by the action of *B. coli* see Sasaki (*Biochem. Zeitschr.*, 1914, **59**, 429). This base has been isolated in various ways. It is hardly soluble in chloroform and in ether, but may be shaken out from a sodium carbonate solution by amyl alcohol, but not from a solution in moderately strong sodium hydroxide; thus it is readily separated from non-phenolic bases (Barger and Walpole). The

fact that the base is very little soluble in ether, but soluble in amyl alcohol, was also used by Gautier, who crystallised the free base and thus separated it from the homologous "*Tyrosamines*," C_7H_9ON and $C_9H_{13}ON$. It is more convenient, however, to benzoylate by the Schotten-Baumann method; the resulting dibenzoyl-derivative, m. p. 170° , crystallises readily from alcohol (Emerson, Langstein, Rosenheim, Barger and Walpole). The base has also been isolated as the platinichloride, after preliminary precipitation as phosphotungstate (Winterstein and Küng).

Tyramine was obtained in small quantities long ago by heating tyrosine (Schmitt and Nasse, *Annalen*, 1865, **133**, 214). By heating tyrosine in diphenylamine to 240° a 90% yield of tyramine may be got (Abderhalden and Gebelein, *Z. physiol. Chem.*, 1926, **152**, 125). This is the most convenient method of preparation. Tyramine can also be prepared by the reduction of *p*-hydroxyphenylacetonitrile (Barger, *J. Chem. Soc.*, 1907, **95**, 1127). It has further been synthesised in various other ways (Barger and Walpole, *ibid.*, 1909, **95**, 1720; Rosenmund, *Ber.*, 1909, **42**, 4782). Tyramine crystallises from alcohol in hexagonal leaflets, m. p. 161° . It is soluble in 95 parts of water at 15° (Gautier), 10 parts of boiling alcohol, and somewhat less in boiling water. It is slightly soluble in boiling xylene, but almost insoluble in the cold; xylene is a convenient solvent for recrystallisation. The base is, however, most readily purified by distillation, b.p. 161 to 163° under 2 mm. pressure. The base, like tyrosine, gives Millon's reaction and Mörner's reaction. The dibenzoyl derivative, $C_6H_5.CO.O.C_6H_4.CH_2.CH_2NH.CO.C_6H_5$, melts at 170° and gives Mörner's reaction, but not Millon's reaction. Tyramine is not poisonous, but nevertheless has marked physiological activity (Dale and Dixon, *J. Physiol.*, 1909, **39**, 25). It resembles adrenaline, to which it is also related chemically, and it is the chief cause of the *pressor* action of aqueous ergot extracts. It also acts on the uterus, and was probably the chief cause of the activity of the placental extracts investigated by Dixon and Taylor (*Brit. Med. J.*, 1907, [ii], 1150). Given by the mouth in doses of 30-100 mg. the base produces in man a slight rise of blood pressure lasting for some hours. The effect is much smaller than on intravenous injection, because the liver decomposes tyramine to *p*-hydroxyphenylacetic acid (Ewins and Laidlaw, *J. Physiol.*, 1910, **41**, 78).

B. Derivatives of Diamino-acids

Tetramethylenediamine, Putrescine, $\text{NH}_2\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{NH}_2$, is formed from ornithine, $\text{NH}_2\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{CH}(\text{NH}_2)\text{COOH}$, by putrefaction (Ellinger, *Z. physiol. Chem.*, 1900, **29**, 334; Ackermann, *ibid.*, 1909, **60**, 482). Since ornithine is itself a decomposition product of arginine, putrescine is of common occurrence in the putrefaction of proteins. It was first found by Brieger in putrid material of various kinds. Udranszky and Baumann (*Ber.*, 1888, **21**, 2938) identified it as tetramethylenediamine previously synthesised by Ladenburg, by the reduction of ethylene dicyanide. These authors also obtained putrescine from the urine in cases of cystinuria and isolated it as the dibenzoyl derivative (*Z. physiol. Chem.*, 1889, **13**, 562). It is present in ergot (Rieländer, *Sitzber. Gesellsch. Naturw. Marburg*, August 5, 1908) and in cheese (Winterstein and Thöny, *Z. physiol. Chem.*, 1902, **36**, 28). Putrescine is a liquid, boiling at 156 to 157° , with a piperidine-like odour, also somewhat resembling that of semen. The *dihydrochloride* crystallises in needles, insoluble in absolute alcohol, and only slightly soluble in 96% alcohol (*separation from cadaverine hydrochloride*). The *dipicrate*, m. p. 250° , is sparingly soluble in water; the *dipicolonate* is sparingly soluble in water and in alcohol, m. p. 263° . The *dibenzoyl derivative* melts at 175° , and is sparingly soluble in alcohol.

δ -Amino-*n*-valeric acid, was found by E. and H. Salkowski (*Ber.*, 1883, **16**, 1191) in the putrefaction products of fibrin. It probably arises from ornithine (α - δ -diaminovaleric acid) by the elimination of the α -amino group, and is thus derived from the same parent substance as putrescine. δ -Amino-*n*-valeric acid is a much stronger base than the α -amino acids, such as α -amino-*isovaleric* acid (valine), with which it is isomeric. The *hydrochloride* forms acicular crystals; the *aurichloride* (m. p. 100°) and *platinichloride* are crystalline.

Pentamethylenediamine, Cadaverine, $\text{NH}_2\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{NH}_2$, a homologue of putrescine, is formed from lysine or α - ϵ -diamino-*n*-caproic acid (Ellinger, *Z. physiol. Chem.*, 1900, **29**, 334; Ackermann, *id.*, 1909, **60**, 482). It was discovered by Brieger in putrid horse meat and other putrid material; it was identified as pentamethylene diamine by Ladenburg, and has frequently been found accompanying putrescine. Thus, together with its lower homologue it was found in ergot (Rieländer, *Sitzber. Gesellsch.*

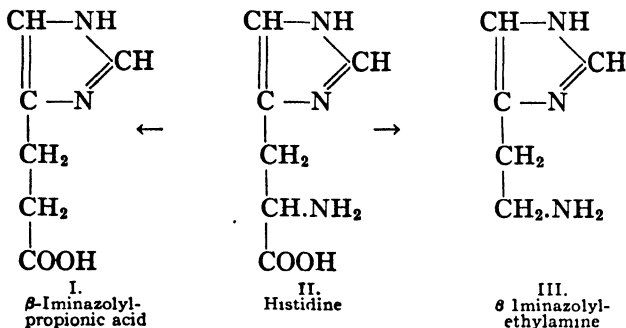
Naturw. Marburg, August 5, 1908), in cheese (Winterstein and Thöny, *Z. physiol. Chem.*, 1902, **36**, 28) and in the urine in cystinuria (Baumann and Udranszky, *Z. physiol. Chem.*, 1889, **13**, 562; Löwy and Neuberg, *id.*, 1905, **43**, 338).

Cadaverine can be transformed into piperidine (Ladenburg, *Ber.*, 1887, **20**, 2216), and conversely it is most conveniently obtained from piperidine, by von Braun's method (*Ber.*, 1904, **37**, 3583). Cadaverine is a syrupy liquid, sp. gr. 0.9174 at 0°, b. p. 175 to 178°, with an odour resembling that of piperidine and of semen. The *dihydrochloride* forms prismatic crystals readily soluble in 96% alcohol (*separation from putrescine hydrochloride*) and yields a *mercury* double salt, $C_5H_{14}N_2 \cdot 2HCl \cdot 4HgCl_2$, m. p. 214°. The *platinichloride*, $C_5H_{14}N_2 \cdot H_2PtCl_6$, is soluble in 70.8 parts of water at 21° and melts at about 215° (Gulewitsch, *Z. physiol. Chem.*, 1894, **20**, 287). The *aurichloride*, $C_5H_{14}N_2 \cdot (HAuCl_4)_2$, forms prisms, fairly readily soluble in water, m. p. 186–188°. The *acid oxalate*, $C_5H_{14}N_2 \cdot 2C_2H_2O_4 + H_2O$, leaflets, m. p. 143°, and the *normal oxalate*, $C_5H_{14}N_2 \cdot C_2H_2O_4 \cdot 2H_2O$, needles, m. p. 160°, both crystallise from dilute alcohol and are insoluble in absolute alcohol.

The *dipicrate*, $C_5H_{14}N_2 \cdot 2C_6H_3O_7N_3$, crystallising in needles, m. p. 221°, and the *dipicrolonate*, $C_5H_{14}N_2 \cdot 2C_{10}H_8O_5N_4$, m. p. 250°, are sparingly soluble in water, the latter also in alcohol. The *dibenzoyl derivative*, $C_5H_{10}(NH.CO.C_6H_5)_2$, melts at 130° and is more soluble in a mixture of alcohol and ether than the corresponding putrescine derivative.

Cadaverine, like putrescine, is not toxic. On intravenous injection it lowers the blood pressure, 10 mg., producing a marked effect in the cat (Barger and Dale, *J. Physiol.*, 1910, **41**, 28). A number of bases (saprine, gerontine, neuridine) have been described, which were considered by their discoverers to be isomeric, but not identical with cadaverine. The two last named are identical with spermine, $C_{10}H_{26}N_4$, a base which occurs as such in the tissues and is not a product of putrefaction (Dudley and Rosenheim, *Biochem. J.*, 1925, **19**, 1634).

Histamine, β -Iminazolyethylamine (III), 4- β -aminoethylglyoxaline, was first obtained by Windaus and Vogt (*Ber.*, 1907, **40**, 3691) from iminazolypropionic acid (II) which has been synthesised, but is also a degradation product of histidine (I).



Subsequently Ackermann (*Z. physiol. Chem.*, 1910, **65**, 504) obtained the base directly by putrefaction of histidine, from which it is derived by elimination of carbon dioxide. Ackermann's yield was 42% of the theoretical and may even be exceeded, if a pure culture of the right organism is available. For optimal conditions see Hanke and Koessler (*J. biol. Chem.*, 1921, **50**, 131). The bacterial method is used industrially. Small quantities of the base can also be obtained by heating histidine with mineral acids (Ewins and Pyman, *J. Chem. Soc.*, 1911, **99**, 339), but the amine is best obtained by synthesis, by Pyman's method (*J. Chem. Soc.*, 1911, **99**, 668).

Histamine has been isolated by Barger and Dale (*J. Chem. Soc.*, 1910, **97**, 2592) from ergot, of which it is one of the chief active principles, and it has also been found to be present in the fresh mucous membrane of the small intestine of the ox (Barger and Dale, *J. Physiol.*, 1911, **41**, 499), in the human intestinal contents (Mutch. *Quart. J. of Med.*, 1914, **7**, 427), and in the contents of the human colon and caecum (Meakins and Harington, *J. Pharm. exp. Therap.*, 1921, **18**, 455; 1922, **20**, 45). The substance was obtained from (fermented) soya beans by Yoshimura (*Biochem. Zeit.*, 1910, **28**, 16) and is probably also present in small quantities in commercial extracts of meat, of yeast, and of other materials. According to Abel and Kubota (*J. Pharm. exp. Therap.*, 1919, **13**, 243) it is present in minute quantity in various tissues, and Best, Dale, Dudley and Thorpe (*J. Physiol.*, 1927, **62**, 379) isolated 27 mg. per kilo. from fresh lung; they discuss its physiological significance.

The *dihydrochloride* crystallises from alcohol in prisms and leaflets, m. p. 240°, and is very slightly soluble in cold absolute ethyl alcohol. The *platinichloride*, $\text{C}_6\text{H}_9\text{N}_3.\text{H}_2\text{PtCl}_6$, forms orange-

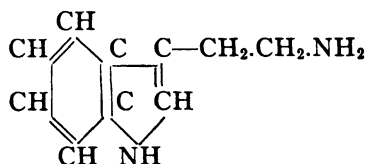
coloured prisms, readily soluble in water, but hardly at all in alcohol, and decomposing above 200° without melting. The *dipicrate* and the *dipicrolonate* are the most characteristic salts. The former, $C_5H_9N_3 \cdot 2C_6H_3O_7N_3$, is very sparingly soluble in cold water and, when separated slowly, forms deep yellow, rhombic leaflets, decomposing at about 235° . The *dipicrolonate*, $C_5H_9N_3 \cdot 2C_{10}H_8O_6N_4$, only dissolves in about 450 parts of boiling water and crystallises in needles, decomposing at about 261° .

Histamine, like histidine, gives an intense red coloration with a solution of *p*-diazobenzene-sulphonic acid in sodium carbonate (Pauly's reaction). It can, however, be distinguished from histidine, in that it does not react with triketohydrindene (Ruhemann's reagent for α -amino acids).

The physiological properties of histamine have been investigated by Dale and Laidlaw (*J. Physiol.*, 1910, **41**, 318 and 1911, **43**, 182) and by many later authors (see Guggenheim's *Biogene Amine*).

Agmatine, Guanidylbutylamine, $NH_2.C(:NH)NH.CH_2.CH_2-CH_2.CH_2.NH_2$, was obtained by Kossel from herring spawn (*Z. physiol. Chem.*, 1910, **66**, 257) and soon after by Engeland and Kutscher (*Zentralbl. f. Physiol.*, 1910, **24**, 479) from ergot. It has not yet been obtained as a putrefaction product, although it might well be so formed (from arginine, by loss of carbon dioxide). It probably breaks down further to putrescine, from which Kossel prepared it synthetically by the action of cyanamide.

Indole ethylamine (3- β -aminoethylindole), was obtained both



synthetically and from tryptophane by putrefaction (Ewins and Laidlaw, *Proc. Chem. Soc.*, 1910, **26**, 343, Ewins, *J. Chem. Soc.*, 1911, **99**, 270). It is formed by *B. mesentericus* from casein (Grimmer and Wiemann, *Forsch. a. d. Geb. d. Milchwirtsch. u. d. Molkereiw.*, 1921, **1**, 2) and was isolated in minute quantity from the urine in pellagra (Sullivan, *J. biol. Chem.*, 1922, **50**, 39).

The base is best prepared by heating γ -amino-butyrylactal and phenylhydrazine with zinc chloride. On removal of the zinc by

hydrogen sulphide and concentration of the solution the hydrochloride crystallises. The free base, $C_{10}H_{12}N_2$, melts and decomposes at 145 to 146° , and forms long colourless needles, readily soluble in alcohol and in acetone, but hardly at all in water, ether, benzene or chloroform. The substance gives the blue-violet coloration with glyoxylic and sulphuric acids, described by Hopkins and Cole (*Proc. Roy. Soc.*, 1901, **68**, 21) as characteristic of tryptophane (Adamkiewicz's reaction). The base still gives this reaction at a dilution of 1:300,000. Unlike tryptophane, it does not give a pink coloration with bromine water.

The *hydrochloride*, $C_{10}H_{12}N_2.HCl$, melts at 246° and dissolves in 12 parts of water at room temperature.

The *picrate*, $C_{10}H_{12}N_2.C_6H_3O_7N_3$, separates at once on the addition of a saturated aqueous solution of picric acid and forms dark red crystals in fern-like aggregates, resembling ammonium chloride crystals. It is almost insoluble in water, very sparingly soluble in alcohol; m. p. 242 to 243° . The *picrolonate* melts at 231° .

The physiological properties of indolethylamine have been described by Laidlaw, *Biochem. J.*, 1911, **6**, 141. Prepared as a fission product of the alkaloid evodiamine, it has been used medicinally in Japan (*Abstr. J. Chem. Soc.*, 1921, **1**, 48).

II. Putrefaction Bases Not Directly Derived from Amino-acids

A. Bases of Known Constitution

Dimethylamine was found by Bocklisch in herring brine, by Brieger in putrid gelatin and in putrid yeast, and by Ehrenberg (*Z. physiol. Chem.*, 1887, **11**, 239) in poisonous sausages.

Trimethylamine, $N(CH_3)_3$, is a very common putrefaction product, occurring for instance in herring brine and in aqueous extracts of ergot. It is not formed in the decomposition of proteins, but results from choline, itself a fission product of lecithin. Trimethylamine is best separated by steam distillation and isolated as the aurichloride, $(CH_3)_3N.HAuCl_4$, m. p. 223 to 226° , sparingly soluble in alcohol and in water.

It has been shown by Tankard (*Analyst*, 1926, **51**, 565) that the trimethylamine occurring in fish may undergo oxidation with the production of a minute amount of formaldehyde— $2(CH_3)_3N + O_2 = 2(CH_3)_2NH + 2HCHO$, and $2(CH_3)_2NH + O_2 = 2CH_3.NH_2 + 2HCHO$. Fish exposed to the air until putrefaction occurs does

not give an increased reaction in Schryver's test for formaldehyde; on the contrary the reaction tends to disappear with the advance of decomposition.

n-Propylamine, found in gelatin cultures of faecal bacteria (Brieger, *Ber.*, 1887, 20, 797) is possibly trimethylamine.

Butylamine and **hexylamine** were found (together with *iso*-amylamine) by Gautier and Mourgues in cod-liver oil (*Compt. Rend.*, 1888, 107, 110).

Neurine, $(\text{CH}_3)_3\text{N}(\text{OH})\cdot\text{CH}=\text{CH}_2$, was found by Brieger in putrid meat (*Ber.*, 1883, 16; 1884, 17) (for description see Index).

Choline, $(\text{CH}_3)_3\text{N}(\text{OH})\text{CH}_2\cdot\text{CH}_2\text{OH}$, is readily formed in putrefaction by the decomposition of lecithin. For description see Index.

Indole and **skatole** occur regularly in the putrefaction of most proteins, and must be derived from tryptophane. (See Index.)

Methylguanidine, obtained by Brieger from putrid meat, might arise from creatine. Its alleged occurrence in muscle is due to oxidation of creatine by the silver salts used to isolate it. (Ewins, *Biochem. J.*, 1916, 10, 103.) Nor does it occur in the urine in tetany as has often been alleged (*cf.* Greenwald. *J. Biol. Chem.*, 1924, 59, 329).

B. Bases of Unknown Constitution

In the case of the large number of other putrefaction bases, which have been described, the constitution is unknown, and in many cases the empirical formula is doubtful. Only the better known ones will be mentioned.

Hydrolutidine, $\text{C}_7\text{H}_{11}\text{N}$, isolated by Gautier and Mourgues from cod-liver oil is a liquid, b. p. 199° . It is chiefly of interest on account of its oxidation with potassium permanganate to methylpyridine-carboxylic acid. Thus the base appears to be dimethyldihydropyridine. Similarly a base of the composition $\text{C}_8\text{H}_{11}\text{N}$, obtained by Oechsner de Coninck (*Compt. rend.*, 1888, 106, 858 and 1604; 1889, 108, 58 and 809) from putrid cuttle fish was oxidised by permanganate to nicotinic acid, which on distillation with lime yielded pyridine. These two are the only putrefaction bases for which experimental evidence of the existence of a pyridine ring has been advanced. The evidence of such a ring was frequently assumed by early investigators and was doubtless suggested by its occurrence in vegetable alkaloids.

Sepsine, $C_5H_{14}O_2N_2$, was obtained in minute quantity from putrid yeast by Faust (*Arch. exp. Path. Pharm.*, 1904, **51**, 248). The base is chiefly characterised by its physiological effects; on intravenous injection it produces in dogs vomiting, diarrhoea, coma and death within 12 hours, without convulsions. The isolation depends on the precipitation of the base by mercuric chloride in the presence of sodium carbonate, and the formation of a crystalline precipitate of the sulphate, when alcoholic sulphuric acid is added to an alcoholic solution of the base. The sulphate, $C_5H_{14}O_2N_2.H_2SO_4$, which was analysed, forms matted needles, which on repeated evaporation of the solution are stated to be transformed into cadaverine sulphate by loss of oxygen. The base is also produced in cultures of *Bacterium sepsinogenes* on agar and in broth.

The formula for sepsine does not appear to be established beyond all doubt.

Viridinine, $C_8H_{12}O_3N_2$, obtained from putrid pancreas by Ackermann (*Z. physiol. Chem.*, 1908, **57**, 28) according to Ackermann and Kutscher's method. The *platinichloride*, $(C_8H_{12}O_3N_2)_2H_2PtCl_6$, forms yellow needles decomposing at 212 to 216°. The *aurichloride*, $C_8H_{12}O_3N_2.HAuCl_4$, crystallises in brownish-black needles, m. p. 176°. The *hydrochloride* forms green needles. Viridine is a mon-acid base.

Marcitine, $C_8H_{12}N_3$, was also obtained from putrid pancreas, by Ackermann (*Z. physiol. Chem.*, 1907, **54**, 204) by the same method. The *aurichloride*, $C_8H_{12}N_3.2HAuCl_4$, melts at 175 to 178°.

Putrine, $C_{11}H_{26}O_3N_2$, was isolated together with marcitine. The *aurichloride*, $C_{11}H_{26}O_3N_2.2HAuCl_4$, forms orange-red crystals melting at 109 to 110°.

The following three bases were obtained by Brieger from cultures of pathogenic bacteria; they should not be confused with the much more complex toxins, which are not thermostable and are neutralised by anti-toxins.

Tetanine, $C_{13}H_{30}O_4N_2$, was obtained by Brieger by inoculating pure beef-broth with the tetanus bacillus, and was found also in human corpses. It is a strongly alkaline yellow syrup, permanent in alkaline solutions but readily decomposing in presence of acids. The hydrochloride is very deliquescent. The *platinichloride*, $C_{13}H_{30}O_4N_2.H_2PtCl_6$, is soluble in water and in alcohol, but is precipitated from its solution in the latter solvent by adding ether. It crystal-

lises in plates which decompose at about 197° . Tetanine is very poisonous, producing tetanic convulsions and death.

Tetanotoxine, $C_8H_{11}N$, is a base extracted by Brieger from cultures of the tetanus bacillus, together with tetanine, $C_{13}H_{30}O_4N_2$, and spasmotoxine, a base of unknown composition. Tetanotoxine is described as a colourless liquid of disagreeable odour, which can be distilled either alone or with steam. The *hydrochloride* melts at about 205° , and dissolves in water and in alcohol. The *platini-chloride* is difficultly soluble and decomposes at 240° , while the *aurichloride* melts at 130° and is readily soluble. Injected subcutaneously, it produces tremor and paralysis, followed by violent convulsions. Isomeric with piperidine.

Typhotoxine, $C_7H_{17}O_2N$, produced by the typhoid bacillus, has been described by Brieger. It is a strong base of alkaline reaction, and possesses poisonous properties. The *aurichloride* forms prisms melting at 176° . The hydrochloride and phospho-tungstate are crystallisable. An isomeric *base* was found by Brieger in horse-flesh which had been putrefying four months. It was poisonous, formed no picrate, but gave a *platinichloride*. The *aurichloride* formed needles or plates melting at 176° and sparingly soluble in water.

Tyrotoxicon is the name given by Vaughan (*Z. physiol. Chem.*, 1886, 10, 146) to a very poisonous crystalline substance extracted by ether from cheese, stale milk and ice-creams. Unfortunately nothing is known about the chemical composition or properties of this substance; it is not even certain whether it is a base.

For the detection of tyrotoxicon in *milk* and *cheese*, V. C. Vaughan recommended the following process (*Analyst*, 8, 14): The filtrate from the curdled milk, or the filtered cold-water extract of cheese, is neutralised with sodium carbonate, transferred to a separator, and shaken with its own volume of pure ether. The mixture is allowed to stand for 24 hours, or until separation is effected, when the ethereal layer is allowed to evaporate spontaneously in an open dish. The residue is dissolved in water, the liquid again shaken with ether, and the ethereal layer separated and allowed to evaporate as before. Repeated extractions with ether should be avoided, as the purer the tyrotoxicon becomes the less readily is it dissolved.

BIBLIOGRAPHY

See the footnote on p. 194.

ANIMAL BASES

BY K. GEORGE FALK

The development of structural organic chemistry has brought about systems of classification of many organic compounds based upon certain well-defined principles. These classifications frequently differ widely from those used earlier at times when the sources of these same compounds, and not their definite chemical properties, were the main facts known. The contents of a chapter on "Animal Bases" are, therefore, not determined alone by certain substances of basic character which are found among the products derived from animal sources, nor by all substances or derivatives of basic character obtained from such sources. It must necessarily comprise a group of substances chosen more or less arbitrarily, limited on the one hand by the fact that a number of compounds which should be included are obtained from other sources, possibly of vegetable origin, and are therefore better considered in such connection, and on the other hand by the impossibility of considering every substance which has been described and which would be included in any given definition of the group under consideration which might be proposed. Further, the work of the synthetic chemist has brought to light many compounds similar to those which have been obtained from natural sources, and which, in a complete treatise, should be included in a given class or type.

Such considerations, applied to plant and animal bases, to natural and synthetic bases, to chemical changes in substances in animals, in plants, and in bacteria, show the undesirability, if not the impossibility, of attempting a strict classification. To put the matter concretely, a given type of substance is to be considered in this chapter, but the individual substances to be actually discussed will be selected mainly as a matter of convenience of presentation, here and in the other volumes of this treatise.

The substances to be described are all derived from ammonia in which one or more of the hydrogen atoms of the ammonia molecule are replaced by substituted as well as unsubstituted hydrocarbon groups. Because no limitation is set upon the nature of the hydrocarbon groups and their substituents, and because more than one substituted ammonia residue may be present in a molecule of the substance, the complexities which may occur are obvious. It is manifestly possible to consider only a limited number of the more important of such substances, even of those which have been isolated from animal sources only.

The order in which the various groups of substances were described in the chapter on Animal Bases in the former editions of this work will be adopted here. The general classification, including nearly all the animal bases of known constitution, is as follows:

1. Pyridine Derivatives; bases specially characteristic of putrefactive change.

2. Monamines; including methylamine, trimethylamine, and other similar bases.

3. Diamines; including piperazine, and certain bases observed to be produced in the putrefactive decomposition of proteins.

4. Amino-acids; including glycocoll, leucine, tyrosine, asparagine, etc.

5. Betaïnes, a special class of amino-bases; including betaïne, choline, neurine, muscarine, etc.

6. Urea and its analogues and derivatives.

7. Imino-bases; including guanidine, glycocyamine, creatine, creatinine, histidine, etc.

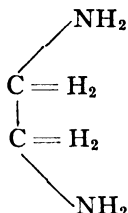
8. Purine or Alloxuric bases; including xanthine, hypoxanthine, guanine, adenine, etc.

The most important bases of the first two classes have already been considered in Vol. VI. Further information respecting some of them will be found under "Putrefaction Bases," as will also descriptions of numerous bases of which the constitutions are not yet known. The diamines of interest which were not described in Vol. V, are considered below; the bases of classes 4 to 8 are then described; and the putrefaction bases as a class are considered in a separate section.

DIAMINES

The diamines are a class of bases derived from two molecules of ammonia by the replacement of two or more of the hydrogen atoms by hydrocarbons of the olefine, phenylene or naphthylene series. The diamines of phenylene, tolylene, and naphthylene have been described in Vol. VI. Piperazine (which has the constitution of a diethylene-diamine) and spermine, are described below. Heuridine, cadaverine, and other putrefaction products having the constitution of diamines are considered under "Putrefaction Bases." Tyramine and histamine will be discussed under amino acids in connection with tyrosine and histidine, from which they are derived.

ETHYLENE-DIAMINE



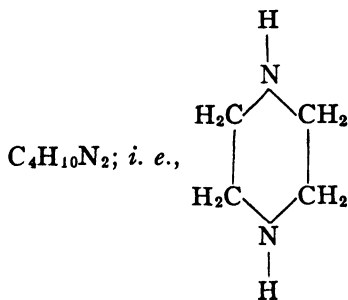
Ethylene-diamine is formed, together with several allied bases, by the reaction of ethylene bromide and alcoholic ammonia at 100° . After cooling, the liquid portion is decanted from the ammonium bromide, evaporated to dryness, and distilled with potassium-hydroxide. The distillate is digested with solid potassium hydromide to absorb water, and the bases separated by fractional distillation. In the manufacture of choral a by-product is obtained which can be conveniently used for the preparation of ethylene-diamine. The fraction of this product boiling between 70° and 100° contains ethylene and ethylidene chlorides, together with higher substitution-products. On treatment with alcoholic ammonia at $100-120^\circ$, the first two substances are converted into diamines. When the reaction is complete, the liquid is poured off from the separated ammonium chloride, and the unaltered chlorides distilled off. From the liquid left in the retort the hydrochloride of ethylene-diamine separates out, and is obtained in silver-white needles after repeated recrystallisation and washing with alcohol.

If the brown mother-liquid is distilled with potassium hydroxide, and the first fraction of the distillate treated with hydrochloric acid, a further crop of crystals of ethylene-diamine hydrochloride will be obtained, while the fractions subsequently distilling contain the higher diamines, triamines, etc.

On distilling with potassium hydroxide the hydrochloride thus prepared, a hydrate of ethylene-diamine is obtained of the composition $C_2H_4(NH_2)_2 + H_2O$; from this the anhydrous base can only be obtained by repeated distillation over sodium. It is a viscous liquid, having a faint ammoniacal odour and burning taste. It melts at 8° , boils at 116.5° , and dissolves easily in water, to form a strongly alkaline liquid. The hydrate melts at 10° and boils at 118° . A number of derivatives of ethylene-diamine may be prepared by condensation of ω -bromethyl derivatives of secondary aromatic amines ($C_6H_5NR.CH_2.CH_2Br$) and secondary amines to form compounds of the type $C_6H_5NRCH_2.CH_2NR'R''$ (J. v. Braun and E. Müller, *Ber.* 51, 1918, 737).

Ethylene-diamine occurs with other diamines among the products of putrefactive decomposition of proteins (see Putrefaction Bases).

Diethylene-diamine. Piperazine



This substance has the constitution of a hexahydropyrazine, standing in the same relation to pyrazine that piperidine does to pyridine. It may be regarded as piperidine in which the CH_2 group in the γ position has been replaced by NH . It is used extensively as a remedy for gout and rheumatism.

Diethylene-diamine was first obtained by A. W. Hofmann, together with monoethylene-diamine, $(C_2H_4)_4H_4N_2$, as a product of the reaction of ammonia and ethylene bromide.

A number of processes have been described and patented for the preparation of diethylene-diamine. They are now mainly of interest from the historical and theoretical sides. The following method of preparation, as worked out by D. S. Pratt and C. O. Young (*J. Amer. Chem. Soc.*, 1918, **40**, 1428) will answer all requirements.

958 grm. of ethylene bromide, 527 grm. of aniline and 575 grm. of sodium carbonate (anhydrous) are thoroughly mixed and heated for 5-6 hours to gentle boiling in an oil-bath, using a reflux condenser. Stirring is decidedly beneficial and probably essential on a large scale, as the reaction product tends to cake. The presence of soda is necessary, otherwise ethylene bromide combines with aniline vigorously at about 95°, to give diphenyl-ethylene-diamine and hydrobromic acid. The acid so freed unites with aniline to form the salt and liberates sufficient heat to cause charring.

The warm melt is extracted with hot water to remove sodium bromide; this can be recovered readily and used as the source of bromide for ethylene bromide. The fact that ethylene chloride can be condensed with aniline similarly should be noted, an autoclave being used to obtain the necessary temperature. The crude diphenylpiperazine is sufficiently pure for making its dinitroso derivative. Yields of from 90 to 95% are usually obtained.

547 grm. of diphenylpiperazine, dried and powdered, are suspended in 2200 c.c. of cold concentrated hydrochloric acid. With mechanical stirring a saturated solution containing 438 grm. of sodium nitrite is slowly added through a tube reaching to the bottom of the acid. In this way, the loss of nitrous fumes is largely prevented and the formation of tar minimised. Cooling is necessary during the addition of nitrite. The resulting product is filtered off with suction and washed once with cold water. It is probably the hydrochloride of dinitrosodiphenyl piperazine. It decomposes readily on drying, but is quite stable if kept moist.

The moist material was added to a 40% solution containing 1900 grm. of sodium bisulphite, and the suspension warmed, with stirring, to about 80°. The reaction proceeds smoothly, giving a deep reddish-orange solution and considerable suspended matter which is removed by filtration, and discarded. The reddish orange solution is made strongly alkaline with sodium hydroxide and concentrated by distillation. Some ammonia is liberated at first, but the amount appears negligible. The distillation is continued

with superheated steam, the flask being immersed in an oil bath at 160° . Piperazine distils over rapidly with the steam, and is caught in hydrochloric acid.

Micro tests are convenient for determining when to stop the distillation. Traces of piperazine give very characteristic light yellow crystals with platinum chloride solution. These vary according to the concentration, but, in general, are square or rectangular. A drop of the condensate on a microscopic slide is run into a drop of platinum chloride solution. The formation of a precipitate at once indicates that the distillation should be continued. Picric acid may be substituted for platinum chloride, but the resulting crystals are not so characteristic. When no precipitate forms at once, examination under the microscope readily reveals whether more than mere traces of piperazine are coming over.

Another reagent giving characteristic crystals with piperazine is bismuth potassium iodide. The reagent is made by adding enough nitric acid to dissolve suspended bismuth nitrate and sufficient potassium iodide to give a clear red solution. Slight traces of piperazine salt in faintly acid solution give very brilliantly coloured garnet crystals with a drop of this reagent. The form of the crystals varies from compact prisms to the more usual clusters of feathery needles. The double salt is very insoluble and may offer a means of determining piperazine quantitatively.

In one experiment the aqueous distillate, distinctly acid with hydrochloric acid, gave 366 grm. of piperazine hydrochloride on evaporation to dryness. The crude salt may be recrystallised from dilute alcohol or converted into the free base by distilling from sodium hydroxide.

Piperazine forms well-defined, colourless, four-sided, glittering tables, which melt at $104-107^{\circ}$ when heated in capillary tubes; although when the m.p. is determined on larger quantities it is found to be 112° —a difference which is probably due to the extremely hygroscopic nature of the base. Piperazine, as usually met with, boils at about 140° (but at 145° when purified by treatment with sodium) and solidifies, on cooling, to a hard crystalline mass.

Piperazine has a faint, aromatic odour, is practically tasteless, and is neither poisonous nor caustic. It is extremely deliquescent and soluble in water, from which menstruum it crystallises in

glittering quadratic tables. It is deposited from absolute alcohol in large, transparent crystals.

The aqueous solution of piperazine has a strongly alkaline reaction, whilst the solid substance readily absorbs carbon dioxide from the air and is converted into the carbonate, melting at $162-165^{\circ}$.

Majert and Schmidt have described the following series of hydrates of piperazine, that most readily-formed being a hexahydrate, which crystallises from dilute aqueous solutions.

Hydrate	Formula	M. p., ° C.
Monohydrate.....	$C_4H_{10}N_2 + H_2O$	75
Dihydrate.....	$C_4H_{10}N_2 + 2H_2O$	56
Trihydrate.....	$C_4H_{10}N_2 + 3H_2O$	39-40
Tetrahydrate.....	$C_4H_{10}N_2 + 4H_2O$	42-43
Pentahydrate.....	$C_4H_{10}N_2 + 5H_2O$	45
Hexahydrate.....	$C_4H_{10}N_2 + 6H_2O$	48

Piperazine hydrochloride forms snow-white matted needles, containing $B(HCl)_2 + H_2O$. It is deliquescent, and very soluble in water, insoluble in alcohol, tastes like ammonium chloride, and is not poisonous. B, H_2PtCl_2 crystallises in small yellow needles, moderately soluble in hot water, but only very sparingly soluble in hot alcohol. B, H_2HgCl_4 crystallises in concentrically grouped needles, readily soluble in hot water, but reprecipitated on adding alcohol. $B, HAuCl_4$ forms small, yellow, glittering scales.

Minute amounts of piperazine may be detected by the formation of a crystalline precipitate with potassium ferrocyanide in hydrochloric acid solution. Similar precipitates are formed by many of the common alkaloids.

Piperazine urate, $C_4H_{10}N_2, C_5H_4O_3N_4$, is a salt which dissolves in 50 parts of water at $17^{\circ}C$. The solubility of piperazine urate has led to the employment of piperazine hydrochloride in gout and rheumatism, in doses ranging from 0.5 to 3.0 grm. daily. Piperazine passes through the human organism unchanged, and may be found in the urine in a very short time. It may be detected by boiling the urine, filtering from albumin or other precipitate, acidifying the filtrate with hydrochloric acid, concentrating it to a small volume and again filtering. The filtrate is treated with strong sodium hydroxide solution and distilled. In the distillate the piperazine may be recognised by adding potassium-bismuth iodide, when the characteristic bismuth compound is precipitated in garnet-red microscopic

crystals. The picrate of piperazine, because of its characteristic crystalline form, cannot be mistaken for the amorphous precipitate produced by albumin with picric acid. Further, the precipitate of piperazine picrate dissolves on heating, and reappears on cooling, whilst that due to albumin is permanent. The nature of the precipitate may be proved by treating it with hydrochloric acid, removing the picric acid by extraction with ether, and recognising the piperazine by its reaction with potassium-bismuth iodide.

Dinitroso-piperazine, $C_4H_8N_2(ON)_2$, is obtained when sodium nitrite is added to a solution of piperazine hydrochloride containing free hydrochloric acid, and the mixture warmed for a short time. A crystalline substance separates out, which, when purified by crystallisation from boiling water, forms yellowish lustrous plates, melting at 158° and sparingly soluble in cold water or ether, but readily in boiling water or hot ether. Dinitroso-piperazine is not decomposed by boiling with alkali hydroxides, or by cold concentrated hydrochloric or sulphuric acid. It gives a deep blue coloration, after some minutes, with a solution of phenol in concentrated sulphuric acid (Liebermann's reagent).

The quantitative estimation of piperazine may be undertaken either by the formation of the salt with platonic chloride, recrystallised from hot alcohol, or by the formation of the picrate, recrystallised from hot water, and weighing.

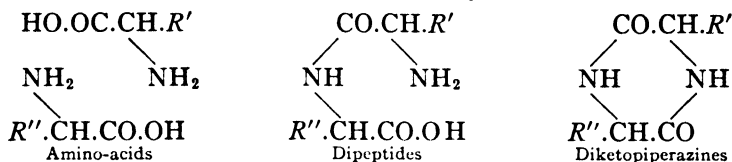
2, 5 Diketopiperazine, $NH \begin{array}{c} \diagup CO-CH_2 \\ \diagdown CH_2-CO \end{array} NH$, is also known as

glycocoll anhydride. It is formed readily from the ethyl ester of glycocoll by heating at 160° , ethyl alcohol being given off (amide formation). It may also be obtained from glycocoll ethyl ester by allowing it to stand in aqueous solution for several days, or very rapidly by the action of alcoholic ammonia or sodium ethylate on glycyglycocoll ethyl ester.

Diketopiperazine crystallises as needles. It is easily soluble in hot water and in dilute alcohol, and insoluble in practically all neutral organic solvents. It melts at 275° , with decomposition if heated slowly, undecomposed if heated rapidly. By the action of cold dilute sodium hydroxide glycyglycine, $NH_2 \cdot CH_2 \cdot CO \cdot NH \cdot CH_2 \cdot CO_2H$, is formed. It forms a silver and a copper salt. Analogous substituted diketopiperazine compounds may be obtained which

are related to, and prepared from, various amino-acids. It may be noted that a number of these diketopiperazines give the Jaffé reaction (red colour) with picric acid and alkali, as does creatinine, although to a less degree, and when sodium carbonate is used in place of sodium hydroxide.

The presence of substituted diketopiperazines as such in proteins has been investigated to a considerable extent, especially in recent years. The relation between amino-acids, dipeptides, and diketopiperazines is brought out by the following formulas:—



The evidence indicates that a small percentage of diketopiperazines occurs in certain proteins, but that the possibility of their formation from various peptides in the acid or other hydrolyses and subsequent treatments of proteins exists and must be guarded against. In view of the lack of certainty which exists in regard to the natural occurrence of these diketopiperazines, other representatives will not be described here. The recent investigations of the composition of proteins, especially in connection with the presence of diketopiperazines as essential constituents have been summarised and reviewed by E. Klarmann (*Chemical Reviews*, 1927, 4, 51) and by A. Hunter (*Trans. Royal Soc. Canada*, May, 1925, 19).

Ethylene-ethenyl-diamine, $\begin{array}{c} \text{CH}_2\text{NH} \\ | \\ \text{CH}_2\text{N} \end{array} \text{C.CH}_3$, has been proposed,

under the name of "lysidine," as a substitute for piperazine in gout. Lysidine is a very hygroscopic, reddish-white, crystalline substance, (melting at 104° and boiling at 223°), having a peculiar odour resembling coniine. It is easily soluble, and is prepared commercially as a 50% aqueous solution, from 2 to 10 grams of which are directed to be taken at a time in aerated water.

Spermine, $\text{NH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NHCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NH}_2$, (α, β -bis[γ' -aminopropylamino]-butane).

Leeuwenhook, in 1678, described the occurrence of "glittering, translucent" crystals in semen and gave their correct measurement.

These crystals were rediscovered by Vauquelin in 1791 and analysed by him. They were then studied by various investigators (Berzelius, Böttcher, and others), but first shown to be the phosphate of a new organic base by P. Schneider in 1878. This phosphate has been stated to be especially plentiful in the spleen, liver, and blood of men and animals suffering from leucocythaemia, and also in the expectorations in cases of bronchial asthma. The chemical structure of this base, spermine, was worked out by O. Rosenheim and his associates (H. W. Dudley and others) in England, while valuable confirmatory evidence was presented by F. Wrede and co-workers in Germany.

Several methods of preparing spermine phosphate have been described. The most satisfactory is the following: 5 c.c. of semen are precipitated by alcohol, and the precipitate filtered and dried. After being finely ground it is dissolved in strong alkali and steam-distilled in a Claisen flask. Distillation is continued until a drop of the distillate no longer reacts with potassium bismuth iodide or picric acid. Altogether 300 c.c. of the distillate are collected and evaporated nearly to dryness. The residue is dissolved in 1 c.c. water, and 0.5 c.c. of a 20% solution of ammonium phosphate is added. Crystallisation begins immediately on scratching with a glass rod, the phosphate depositing as a heavy white powder. (The phosphate may also be prepared by rendering one-third of the solution acid to Congo red by means of dilute phosphoric acid and adding the remaining two-thirds. The resulting solution reacts amphoteric to sensitive litmus paper and deposits well-formed crystals of spermine phosphate on standing. The crystallisation is hastened and completed by the addition of alcohol.) Yield 14 mg. = 0.28%. Important for the isolation is the fact that spermine phosphate crystallises only between pH 6.8 and 7.2. This may be of physiological significance.

The free base is obtained by adding 50% potassium hydroxide solution to a concentrated solution of the hydrochloride or to a water suspension of the phosphate, extracting with chloroform, and evaporating the chloroform *in vacuo*. The spermine remains as a colourless oil which rapidly solidifies to an aggregate of needle-shaped crystals, melting at 55–60°. It boils without decomposition at about 150° under 5 mm. pressure. It is probably present as a hydrate, is easily soluble in water, ethyl alcohol, and butyl alcohol,

and insoluble in ether, benzene and ligroin. On exposure to air it absorbs carbon dioxide rapidly and liquefies.

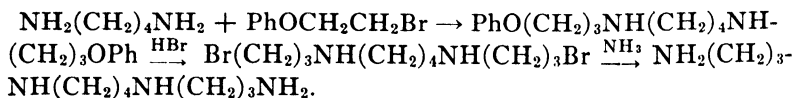
Spermine phosphate is soluble in water to an extent of 1% at 100°, and 0.037% at 20°. When precipitated by the addition of alcohol, slender long needles are formed. Formula, $C_{10}H_{26}N_4 \cdot 2H_3PO_4 \cdot 6H_2O$. The water of crystallisation is lost over sulphuric acid or at 100°, the crystals becoming opaque. They begin to soften at 227°, melt at 230–234° with frothing and evolution of gas, at 240° gas evolution stops and the tube is lined with a white deposit which runs together at 260–262° with a slight evolution of gas. No discoloration occurs during these changes.

Picrate: slender yellow needles, turning black at about 242°, m. p. 248–250° with decomposition. *Hydrochloride*, $C_{10}H_{26}N_4 \cdot 4HCl$: short prismatic needles, turning brown at 300–302°. *Picrolonate*: microscopic short prismatic needles of a dull, pale-yellow colour, m. p. 288–289° with decomposition. *Chloroaurate*: $C_{10}H_{26}N_4 \cdot 4HCl \cdot 4AuCl_3$: golden yellow, lustrous leaflets, m. p. 225°, with decomposition. *Chloroplatinate*, $C_{10}H_{26}N_4 \cdot 2H_2PtCl_6$: large, well-formed crystals, m. p. 242–245° with decomposition. *Benzoyl spermine*, $C_{10}H_{22}N_4 \cdot (CO \cdot C_6H_5)_4$: woolly balls of fine needles, m. p. 155°. *Phenylisocyanate derivative of spermine*, colourless clusters of needles, m. p. 179–180°. *Arsenate*: resembles phosphate in form and solubility. *Oxalate*: leaflets, m. p. 225°. The sulphate, nitrate, acetate, and carbonate crystallise well but are very hygroscopic. A number of additional derivatives of spermine have been prepared. The free base, as well as the hydrochloride, showed no optical activity.

Spermine has been isolated from the following animal organs: testis (yield as spermine phosphate, from bull 0.006%), ovary (cow 0.014%), pancreas (0.025%), muscle, liver, brain (0.007%), spleen (0.011%), thymus (0.006%), thyroid (0.003%). It also has been obtained from yeast (0.01%). Spermine is absent from bull-semen, ox-blood, cow's-milk, and hen's egg.

Analysis of spermine and its derivatives and molecular weight determinations indicated the formula $C_{10}H_{26}N_4$. Methylation and benzoylation showed the presence of two NH_2 and two NH groups. Destructive distillation of the hydrochloride yielded pyrrolidine among the products, while tetramethyltrimethyldiamine was obtained by the degradation of decamethylspermine, proving the

presence of the chains $\text{N}-\text{C}-\text{C}-\text{C}-\text{N}$ and $\text{N}-\text{C}-\text{C}-\text{C}-\text{N}$. The structure was definitely proved by synthesis as follows:—



Spermidine, $\text{NH}_2\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{NH}\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{NH}_2$ α -(γ -aminopropylamino)- β -aminobutane, occurs in the mother liquors after the isolation of spermine from tissue extracts. Its phosphate is much more soluble than that of spermine, and crystallises from 50% alcoholic solution (spermine from 25% alcoholic solution). Two grm. of the phosphate were obtained from 100 kilos. of ox pancreas.

Phosphate, $(\text{C}_7\text{H}_{19}\text{N}_3)_2\cdot 3\text{H}_3\text{PO}_4$: lustrous plates, shrink at 150° , m. p. $207-209^\circ$ to an opaque fluid which begins to froth at $218-220^\circ$ without discolouring. *Picrate*: long thin laminated plates, m. p. $210-212^\circ$ with decomposition. *Hydrochloride*, $\text{C}_7\text{H}_{19}\text{N}_3\cdot 3\text{HCl}$: glistening thin plates. *Chloroaurate*, $\text{C}_7\text{H}_{19}\text{N}_3\cdot 3\text{HAuCl}_3$: thin, lustrous, golden yellow plates, m. p. $220-222^\circ$ with decomposition. *m-Nitrobenzoylspermidine*, $\text{C}_7\text{H}_{16}\text{N}_3(\text{CO}\cdot\text{C}_6\text{H}_4\cdot\text{NO}_2)_3$: balls of radiating needles, m. p. $148-150^\circ$.

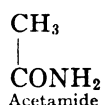
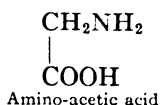
The formula of spermidine was proved by analysis and molecular weight determinations, while the structure was proved by synthesis of the compound.

The properties of spermine and spermidine are very similar. They accompany each other in the methods of isolation from tissue extracts which have been described. Both give the pyrrole reaction, appear in the lysine fraction in the method of Kossel and Kutscher, form phosphotungstates insoluble in acetone, are optically inactive, do not reduce permanganate in faintly acid solution, and yield the characteristic semen-like odour when the solutions of the chloroaurates are treated with magnesium.

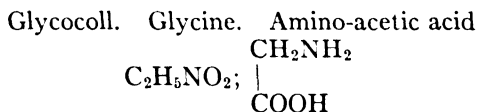
AMINO COMPOUNDS

The compounds which are to be considered in this section include the fatty acids in which a hydrogen of the methyl or methylene group is replaced by the amino (NH_2) or substituted amino group. These are the amino-acids. They differ from the acid amides in that in the latter, the hydroxyl group of the fatty acid is replaced

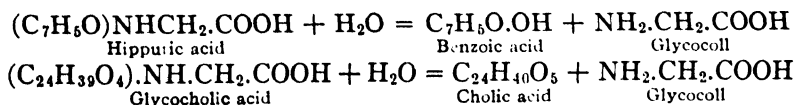
by the amino group, or the hydrogen of ammonia is replaced by an acid radical. The following formulae indicate the relations:



The methods of preparation and properties of the various compounds will be considered in detail in connection with the individual substances. The following table indicates the relations of the various compounds to each other from the structural side and illustrates the general nomenclature in use. In general terms, it may be stated that treatment of a chloro- or bromo-acid with ammonia yields an amino-acid; similar treatment of an acid chloride or anhydride yields an acid amide, as does heating the ammonium salt of the corresponding acid to about 230° , or partial hydrolysis of a suitable cyanide by concentrated acid in the cold, or the interaction of acids or their anhydrides with their nitriles (to form secondary or tertiary amides). Treatment of substances containing the amino group with nitrous acid results in the replacement of the amino group by the hydroxyl group, nitrogen being evolved. In this way, for example, amino-acetic acid (glycocoll) yields hydroxy-acetic acid (glycollic acid); aspartic acid (succinamic acid) yields hydroxy-succinic acid (malic acid); etc.



Glycocoll does not appear to occur frequently ready-formed in nature, though it is said to occur in the muscle of *Pecten irradians*. It is a very frequent product of the action of acids or alkalies on proteins. It was first obtained by Braconnot in 1820 by boiling glue with sulphuric acid, whence its name of glycocoll or sugar of gelatin. It is also formed when hippuric acid is boiled with hydrochloric acid, or when glycocholic acid is boiled with barium hydroxide solution.



Acid	Amino-acid (H replaced by NH ₂)	Amic acid (OH replaced by NH ₂)	Amide (CO.OH replaced by CO.NH ₂)
$\begin{array}{c} \text{CH}_3 \\ \\ \text{COOH} \\ \text{Acetic acid} \end{array}$	$\begin{array}{c} \text{CH}_2\text{NH}_2 \\ \\ \text{COOH} \\ \text{Aminoacetic acid} \\ \text{Glycocol} \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{CO.NH}_2 \\ \text{Acetamide} \end{array}$
$\begin{array}{c} \text{CH}_2\text{OH} \\ \\ \text{COOH} \\ \text{Hydroxyacetic acid} \\ \text{Glycollic acid} \end{array}$	$\begin{array}{c} \text{CH(NH}_2\text{)OH} \\ \\ \text{COOH} \\ \text{Aminoglycollic acid} \end{array}$	$\begin{array}{c} \text{CH}_2\text{NH}_2 \\ \\ \text{COOH} \\ \text{Glycolamic acid} \\ \text{Glycocol} \end{array}$	$\begin{array}{c} \text{CH}_2\text{OH} \\ \\ \text{CO.NH}_2 \\ \text{Glycolylamide} \end{array}$
$\begin{array}{c} \text{CH}_3 \\ \\ \text{CHOH} \\ \\ \text{COOH} \\ \text{Hydroxypropionic acid} \\ \text{Lactic acid} \end{array}$	$\begin{array}{c} \text{CH}_2\text{OH} \\ \\ \text{CHNH}_2 \\ \\ \text{COOH} \\ \text{Aminolactic acid} \\ \text{Serine} \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{CHNH}_2 \\ \\ \text{COOH} \\ \text{Alanine} \end{array}$	$\begin{array}{c} \text{CH}_3 \\ \\ \text{CHOH} \\ \\ \text{CONH}_2 \\ \text{Lactamide} \end{array}$
$\begin{array}{c} \text{OH} \\ \\ \text{C} = \\ \\ \text{OH} \\ \text{Carbonic acid} \end{array}$	$\begin{array}{c} \text{NH}_2 \\ \\ \text{C} = \text{O} \\ \\ \text{OH} \\ \text{Carbamic acid} \end{array}$	$\begin{array}{c} \text{NH}_2 \\ \\ \text{C} = \text{O} \\ \\ \text{NH}_2 \\ \text{Carbamide} \\ \text{Urea} \end{array}$
$\begin{array}{c} \text{COOH} \\ \\ \text{COOH} \\ \text{Oxalic acid} \end{array}$	$\begin{array}{c} \text{CO.NH}_2 \\ \\ \text{COOH} \\ \text{Oxamic acid} \end{array}$	$\begin{array}{c} \text{CO.NH}_2 \\ \\ \text{CO.NH}_2 \\ \text{Oxamide} \end{array}$
$\begin{array}{c} \text{COOH} \\ \\ \text{CH}_2 \\ \\ \text{CH}_2 \\ \\ \text{COOH} \\ \text{Succinic acid} \end{array}$	$\begin{array}{c} \text{COOH} \\ \\ \text{CH}_2 \\ \\ \text{CH.NH}_2 \\ \\ \text{COOH} \\ \text{Aminosuccinic acid} \\ \text{Aspartic acid} \end{array}$	$\begin{array}{c} \text{CO.NH}_2 \\ \\ \text{CH}_2 \\ \\ \text{CH.NH}_2 \\ \\ \text{COOH} \\ \text{Aminosuccinamic acid} \\ \text{Asparagine} \end{array}$	$\begin{array}{c} \text{CO.NH}_2 \\ \\ \text{CH}_2 \\ \\ \text{CH}_2 \\ \\ \text{CO.NH}_2 \\ \text{Succinamide} \end{array}$
$\begin{array}{c} \text{COOH} \\ \\ \text{CH}_2 \\ \\ \text{CHOH} \\ \\ \text{COOH} \\ \text{Malic acid} \end{array}$	$\begin{array}{c} \text{CO.NH}_2 \\ \\ \text{CH}_2 \\ \\ \text{CHOH} \\ \\ \text{CO.NH}_2 \\ \text{Malamide} \end{array}$
$\begin{array}{c} \text{COOH} \\ \\ \text{CHOH} \\ \\ \text{CHOH} \\ \\ \text{COOH} \\ \text{Tartaric acid} \end{array}$	$\begin{array}{c} \text{COOH} \\ \\ \text{CHOH} \\ \\ \text{CHOH} \\ \\ \text{CO.NH}_2 \\ \text{Tartramic acid} \end{array}$	$\begin{array}{c} \text{CO.NH}_2 \\ \\ \text{CHOH} \\ \\ \text{CHOH} \\ \\ \text{CO.NH}_2 \\ \text{Tartramide} \end{array}$

Glycocol may be prepared by boiling hippuric acid for half an hour with 4 parts of fuming hydrochloric acid. The product is diluted with water and allowed to cool, when the greater part of the benzoic acid crystallises out. The remainder is removed by extraction with ether or petroleum spirit, and the solution of glycocol

hydrochloride evaporated till the salt crystallises on cooling. It is washed with absolute alcohol, and on treatment with an equivalent amount of litharge or oxide of silver yields free glycocoll, which is recrystallised from water or dilute alcohol.

Glycocoll may also be prepared by boiling glycocholic acid for twelve hours with strong hydrochloric acid, filtering from the resinous mixture of cholic acid and dyslysin, and evaporating the filtrate. The glycocoll hydrochloride is dissolved in water and treated with lead hydroxide, the liquid filtered, and the soluble lead compound of glycocoll decomposed by hydrogen sulphide. On concentrating the filtered liquid, glycocoll is deposited in crystals.

Glycocoll may also be prepared by boiling a concentrated solution of chloracetic or bromacetic acid with a large excess of strong ammonia.

Glycocoll may be synthesised in quantity as follows (*Organic Syntheses*, 1925, O. Kamm, Editor, Vol. 4):—1500 c.c. of filtered technical formaldehyde (35% by weight) and 540 gm. of ammonium chloride are placed in a 5 litre round-bottomed flask, cooled by an ice mixture, and a solution of 490 gm. of 98% sodium cyanide in 850 c.c. of water added slowly (about 90 drops per minute), with thorough mechanical stirring. The temperature must remain between 0° and 5°. The addition takes about 6 hours. After about half the cyanide has been added (all the ammonium chloride having gone into solution) the addition of 380 c.c. of glacial acetic is started (2 to 2.5 c.c. per minute), so that the acetic acid and cyanide additions are completed at the same time. The mixture is stirred for one and one-half hours longer, the precipitated methylene amino-acetonitrile filtered, transferred to a beaker, stirred with 1.5 litres of water, filtered with the aid of suction, washed with 500 c.c. of cold water (to remove chlorides), and dried on filter paper. Yield 410-475 gm. (64-74% of theory); m. p. of product 129°.



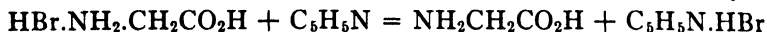
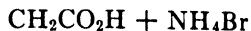
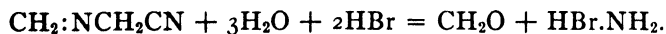
A mixture of 340 gm. methylene amino-acetonitrile and 2500 gm. of 48% hydrobromic acid is heated in a 5 litre flask with return condenser on a steam bath for 3 hours. Acid and formaldehyde are then distilled from the mixture under reduced pressure into a well-cooled receiver until the volume is reduced about one-half. The separated ammonium bromide is filtered from the hot liquid, washed

with a small amount of cold water, the filtrates returned to the flask, and distillation continued almost to dryness. The residue is dissolved in 2 litres of cold methyl alcohol and filtered; to the filtrate are added 300 c.c. of pyridine, with vigorous shaking, the mixture allowed to stand over night, and the precipitated glycoll filtered off and



FIG. 1.—Glycoll (synthetic). $\times 5$. (From Keenan, *J. Biol. Chem.*, 1924, **62**, 163.)

washed with methyl alcohol until the washings are free from bromides. The product is purified by dissolving it in 300 c.c. of boiling water (decolorising carbon) and precipitating at $40-50^{\circ}$ with 1500 c.c. of methyl alcohol, and repeating. The product is filtered when the mixture has cooled, washed with methyl alcohol until free from ammonia (Nessler solution). Yield 115–140 gm. of glycoll; m. p. $225-230^{\circ}$ (decomp.) (31–37% of theoretical amount).



Glycocoll may also be prepared by boiling a concentrated solution of chloracetic or bromacetic acid with a large excess of strong ammonia.

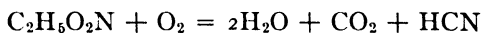
Glycocoll has the constitution of an aminoacetic acid, and is the type of the class of substances of which the proteins are built up.

Glycocoll forms very hard, flattened prisms or aggregated plates, belonging to the monoclinic system (Fig. 1). The crystals grate between the teeth and have a sweet taste, but are not poisonous. Glycocoll melts, with decomposition, at 232–236°.

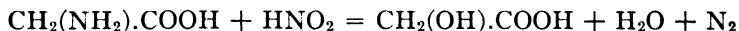
Glycocoll dissolves in 4.3 parts of cold, and in a smaller quantity of boiling, water. It crystallises readily by spontaneous evaporation of its aqueous solution. It is moderately soluble in dilute alcohol, but insoluble in absolute alcohol, even when boiling, as also in ether. It is optically inactive. It is not susceptible of alcoholic fermentation.

When glycocoll is boiled with concentrated alkali hydroxide it evolves ammonia, and, on treating the residue with hydrochloric acid, hydrocyanic acid is disengaged, while oxalic acid is found in the liquid.

Glycocoll is charred by strong sulphuric acid. Distilled with dilute sulphuric acid and manganese or lead dioxide, it yields hydrocyanic and carbonic acids:



Nitrous acid converts glycocoll into glycollic acid, thus:



On agitating the liquid with ether, the glycollic acid is dissolved.¹

Glycocoll evolves no nitrogen when treated with an alkaline solution of sodium hypobromite (A. H. Allen).

On addition of mercurous nitrate, cold solutions of glycocoll yield a grey precipitate of metallic mercury, but the reaction occurs more readily on heating.

¹ On separating and evaporating the solution, the glycollic acid is obtained in fine laminae, which are unchanged in the air, melt at 80°, and are readily soluble in alcohol.

Glycollic acid, $\text{CH}_2(\text{OH}).\text{COOH}$, forms crystallisable salts, most of which are readily soluble. The neutral *lead* salt is soluble in cold water, but, on boiling its solution, or on precipitating a glycollate with lead acetate, a basic salt, $(\text{C}_2\text{H}_3\text{O}_3\text{Pb})_2\text{O}$, separates in cellular needles, requiring 10,000 parts of water for solution. *Cupric glycollate*, $\text{Cu}(\text{C}_2\text{H}_3\text{O}_3)_2$, forms blue crystals, which require 134 parts of cold water for solution.

On addition of ferric chloride to a solution of glycocholl a strong red coloration is produced. This is destroyed by acids, but reappears on cautious neutralisation.

When treated with a drop of phenol and then with a solution of sodium hypochlorite, glycocholl gives a blue coloration, in this reaction resembling ammonia, aniline, and methylamine.

On heating glycocholl in a sealed tube with benzoic acid, hippuric acid is produced. The same product is formed by treating glycocholl with hydrochloric acid and benzoyl chloride. This reaction has been proposed by C. S. Fischer (*Z. physiol. Chem.*, **29**, 164) for the determination of glycocholl, and the value of the method has been confirmed by M. Gonnermann (*Pflüg. Arch.*, 1894, **59**, 42). When glycocholl is taken internally, it appears in the urine as hippuric acid, which has the constitution of a benzoyl-glycocholl, $(C_6H_5CO) \cdot NH \cdot CH_2 \cdot COOH$.

Glycocholl is an amphoteric substance and, under suitable conditions, combines with either bases or acids to form salts. On boiling an aqueous solution of glycocholl with cupric hydroxide or acetate, cupric aminoacetate, $Cu(C_2H_4O_2N)_2 + H_2O$, separates in fine blue needles on cooling or on adding alcohol. The compound dissolves in alkali hydroxide, with deep blue colour.

The barium, strontium, calcium, and magnesium salts of aminoacetic acid have been obtained in a crystalline state (*Annalen*, **266**, 292), and the mercury, lead, cadmium, and palladium salts are likewise crystalline. The silver salt crystallises in tablets, has a strong alkaline reaction, turns grey in the light, and decomposes at 100° .

In addition to the homologues, several important and interesting derivatives of glycocholl occur naturally in the animal kingdom. Among these substances are hippuric and salicyluric acids, occurring in urine; glycocholic acid, a constituent of bile; glycoctamine, etc.

Glycocholl hydrochloride, $C_2H_5O_2N \cdot HCl$, forms deliquescent crystals, having an acid reaction and astringent taste. It is readily soluble in water, but only slightly in alcohol. $B_2 \cdot HCl$ forms trimetric crystals. $B_2 \cdot H_2SO_4$ forms large non-deliquescent prisms, soluble in water, but insoluble in alcohol or ether. $B \cdot HNO_3$ and $B_2 \cdot H_2C_2O_4$ are also crystallisable. Glycocholl phosphotungstate $(C_2H_5ON)_3 \cdot H_3PO_4 \cdot 12WO_3$, usually with 5 or 6 H_2O of crystallisation, forms microscopic whetstone-shaped crystals. The picrolonate melts at 214° , and dissolves in water, 0.99 parts in 100, at $20-23^\circ$.

The copper salt of glycocoll crystallises in fine blue needles. The crystals of the hydrochloride of the ethyl ester melt at 144° . This is the best state in which to isolate glycocoll, and a quantitative estimation may be grounded upon the nitrogen estimation of this salt. The compound with β -naphthalene-sulpho-chloride melts at 159° , the phenyl-isocyanate melts at 195° . The calcium salt of glycocoll-carbamic acid is also characteristic.

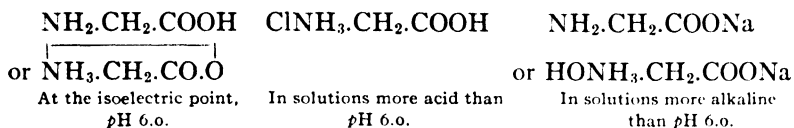
Glycocoll is the lowest member in the series of amino-acids, now recognised as among the most important components of the body. The proteins are composed of amino-acids, condensed with the extrusion of water. When the proteins are hydrolysed, the elements of water are added and the amino-acids set free. These amino-acids are fatty acids in which a hydrogen in the methyl or methylene group is replaced by NH_2 .

The properties of the amino-acids indicate clearly the parts played, and the chemical actions due, to the amino and the carboxyl groups. The simpler amino-acids contain one amino and one carboxyl group, the amino group being attached to the α carbon atom, adjacent to the carboxyl group. Diamino acids contain two amino groups, one α to the carboxyl and one attached to some other carbon atom, and a carboxyl group. Dicarboxylic amino-acids contain two carboxyl groups and one amino group. All these substances are amphoteric; that is, they are capable of combining with acids or with bases. Each may exist in three states. When uncombined with acids or bases an amino-acid (or other amphoteric substance) is said to be in the isoelectric state. Dissolved in water under these conditions, the solution would have a definite hydrogen ion concentration, characteristic for the substance, called the isoelectric point of the substance under discussion. In solutions more acid than this, the substance acts as a base and combines with acids to form salts (as do other substituted ammonias); in solutions more alkaline, the substance acts as an acid and reacts with bases to form salts (as do other substituted fatty acids).

The hydrogen ion concentrations are frequently given in terms of $p\text{H}$ —a simple formulation introduced by Sørensen. The $p\text{H}$ value denotes the negative value of the exponent of 10 representing the hydrogen ion concentration, or is equal to $\log \frac{1}{[\text{H}^+]}$. For

example, $[H^+] = 10^{-5.0}N$ would be written $pH\ 5.0$, etc.¹ These relations, involving exact determinations of acid and alkaline conditions in terms of hydrogen ion concentrations, are becoming increasingly important, as they afford one of the simplest and most satisfactory methods for controlling conditions of reactions in solutions.

The relations outlined may be illustrated with glycocoll as example. Glycocoll, when dissolved, has an isoelectric point of $pH\ 6.0$. It then exists uncombined. In solutions more acid than $pH\ 6.0$ with hydrochloric acid, for example, it would be present as hydrochloride, in solutions more alkaline, as the sodium salt, possibly. This is indicated by the following formulae



As indicated by the formulae, the properties and behaviour of glycocoll is entirely different in solutions more acid than the isoelectric point than in solutions more alkaline than this. For example, in passing an electric current through a solution of glycocoll, at the isoelectric point the glycocoll complex would not travel toward either electrode; in more acid solutions the complex would travel toward the cathode, the ions being Cl^- and $(NH_3CH_2CO_2H)^+$; in more alkaline solutions the complex would travel toward the anode, the ions being $(NH_2CH_2CO.O)^-$ or $(HONH_3CH_2CO.O)^-$ and Na^+ . Such cataphoresis experiments on amino-acids, proteins, and other amphoteric electrolytes (or ampholytes) have thrown much light on the properties of these substances. There is evidently a transition point at the isoelectric point at which the properties of these compounds may change their characters completely. A number of physical and chemical properties have been shown to be readily accounted for with proteins and similar substances on this basis, and a comparatively simple explanation based upon definite chemical relations has been developed to coordinate the large mass of facts available.²

¹ For a complete and satisfactory discussion of these relations, cf. W. M. Clark, "The Determination of Hydrogen Ions," Third Edition, Baltimore, 1928.

² Cf. especially J. Loeb, "Proteins and the Theory of Colloidal Behaviour," Lippincott and Company, Philadelphia, 1922.

These fundamental chemical considerations simplify the study and use of various reactions, both general and individual, of amino-acids, as well as of complex proteins and other amphoteric substances. The chemical properties which are made use of in determining or estimating amino-acids in general involve the changes which the amino group and the carboxyl group may undergo. When a pure amino acid is dissolved in water, the resulting solution has a hydrogen ion concentration which depends on the isoelectric point of the amino-acid. This hydrogen ion concentration is then the true "neutral" point of the solution. It may not be the same "neutral" point as pure water, which is very close to $pH\ 7.0$ ($H^+ = OH^- = 10^{-7.0}N$) at 25° . For glycine it would be $pH\ 6.0$; for aspartic acid (two carboxyl groups and one amino group) $pH\ 3.0$; etc.

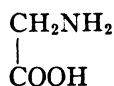
In attempting to titrate such substances with acid or with alkali, the same difficulty is met with as in titrating a solution containing a weak alkali and a weak acid (such as ammonia and acetic acid). The end point with an indicator is not sharp. That is to say, there is no large change in hydrogen ion concentration with the addition of a small quantity of acid and alkali, with the consequent result that the colour change of the indicator is gradual with the small change in hydrogen ion concentration due to added acid or alkali. This is manifestly due to the presence of both the amino and carboxyl groups. It has been proposed to diminish or eliminate the interfering action of the amino group in titrating amino-acids with alkali by the addition of a large quantity of alcohol (R. Willstätter and E. Waldschmidt-Leitz, *Ber.*, 1921, **54**, [B], 2988). A more satisfactory method is Sørensen's formol method, in which the effect of the amino group is eliminated entirely by the addition of formaldehyde to form a Schiff base, leaving the carboxyl group free to be titrated as with a simple fatty acid. This method will be given more in detail following the description of the various amino-acids.

It may be mentioned here that all α -amino-acids, except glycocoll, contain an asymmetric carbon atom (the one to which the NH_2 is attached) and thus may rotate the plane of polarised light. In nature only one optical form usually occurs, the *d* or *l* form as the case may be. But the opposite form can, as a rule, be prepared in the laboratory; and the racemic amino-acids may thus be prepared. The optical rotation is an important analytical property of these

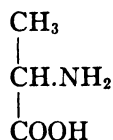
substances. The power of proteins to rotate the plane of polarised light is in part, of course, the expression of the property in the component amino-acids, just as the amphoteric, acid or basic property of native proteins is, in the main, the expression of the NH_2 and COOH groups of the amino-acids. When amino-acids are combined to form proteins, it is, as a rule, through a linkage in which the COOH group of one amino-acid is linked to the NH_2 group of another amino-acid.

There are certain chemical reactions that are common to all the members of the group of amino-acids. These will be first detailed. Then the methods for the isolation and separation of the amino-acids will be described, following which the properties and relations of the individual members of the group will be stated. The isolation of amino-acids may be necessary in the study of digestion mixtures, of the urine and fæces, in the study of germination in plant seeds, and in general in investigations into the protein metabolism of animals and plants. It is also now possible to separate different proteins by the study of their content of the different amino-acids. The following are the important amino-acids which are known to occur in the different native proteins, with their formulae.

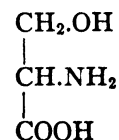
Glycocoll, amino-acetic acid,



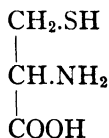
Alanine, α -amino-propionic acid,



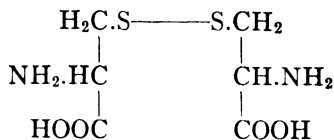
Serine, α -amino- β -hydroxypropionic acid,



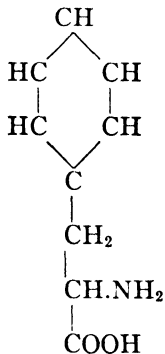
Cystine. This is a double molecule of cystein. Cystein is α -amino- β -thio-propionic acid,



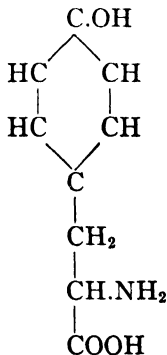
Cystine has therefore the formula



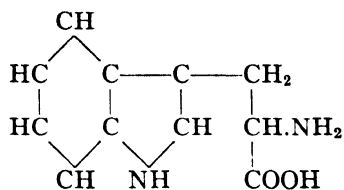
Phenylalanine, Phenyl- α -amino-propionic acid,



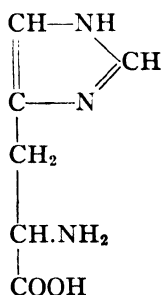
Tyrosine, hydroxy-phenyl- α -amino-propionic acid,



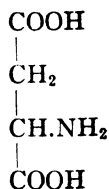
Tryptophane, Indole- α -amino-propionic acid,



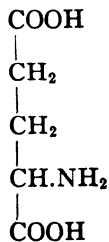
Histidine, α -amino- β -iminazolypropionic acid,



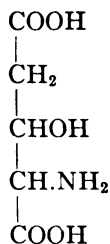
Aspartic acid, α -amino-succinic acid,



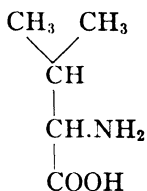
Glutamic acid, α -amino-glutaric acid,



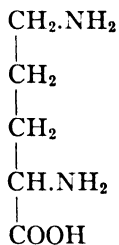
β -Hydroxyglutamic acid, α -amino- β -hydroxy-glutaric acid,



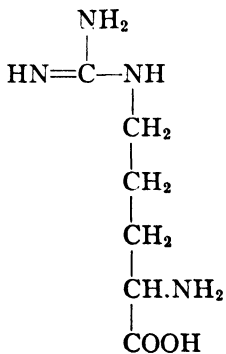
Valine, α -amino-isovaleric acid



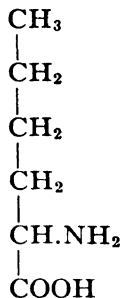
Ornithine, α - δ -diamino-valeric acid,



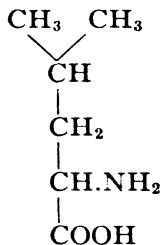
Ornithine does not exist preformed, but only combined with urea to form arginine:



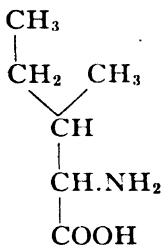
Nor-leucine, α -amino-caproic acid,



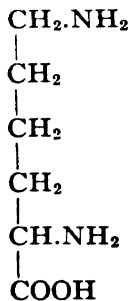
Leucine, α -amino-isobutyl-acetic acid,



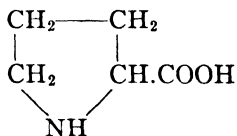
Iso-leucine, α -amino- β -methyl- β -ethyl-propionic acid,



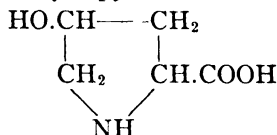
Lysine, α - ϵ -diamino-caproic acid,



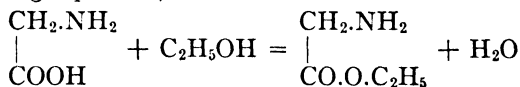
Proline: This is a heterocyclic amino-acid, α -pyrrolidine-carboxylic acid,



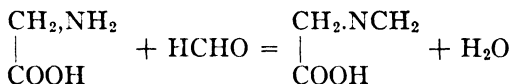
Hydroxyproline, γ -hydroxy- α -pyrrolidine-carboxylic acid,



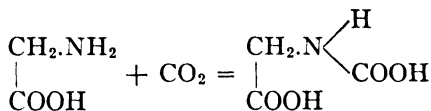
Certain reactions are common to these amino-acids. They combine with alcohols to form esters. Since the hydrogen ion is then no longer present, these substances act as bases. Thus



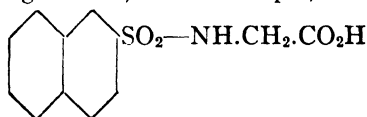
They combine with aldehydes to form methylene compounds that are acids. This is the basis of Sørensen's formol method for estimating amino-acids. Thus



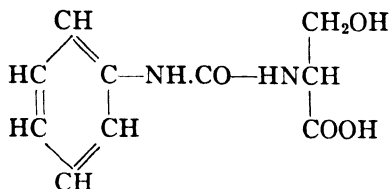
When carbon dioxide is fixed to the amino-group carbamino acids are formed.



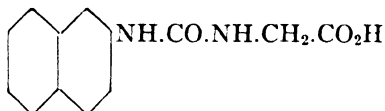
Amino-acids, and hydroxyamino-acids and peptides as well, combine with β -naphthalene-sulpho-chloride to form comparatively insoluble compounds with definite crystallographic properties and sharp melting points. The chlorine is replaced, and the amino-acid is attached to the SO_2 by the amino-group, substituted sulphonamides being formed, as for example, with glycoll:—



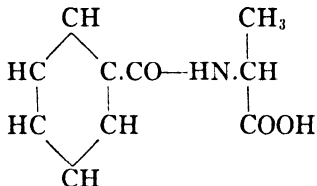
Amino-acids combine with phenyl isocyanate, to form characteristic crystalline compounds. This may be illustrated for serine as follows:—



With α -naphthyl-isocyanate, amino-acids combine to form well-defined crystalline substances; for example, with glycine:—



Benzoyl chloride yields with amino-acids stable compounds, the chlorine being replaced. The benzoyl derivative of alanine may be given as example:—



Picrolonic acid forms, especially with diamino-acids and diamines, well-defined compounds, the mode of attachment being not yet clear. Many of them are insoluble in cold water, but much more readily soluble in alcohol.

Separation of Amino-acids

The problem of the estimation of amino-acids may arise under one of two circumstances. A material, such as intestinal contents, urine, a bacterial culture medium or autopsy organs, may require analysis for the presence of preformed amino-acids. On the other hand, one may wish to determine the component amino-acids of a protein. In the first instance one proceeds directly with the isolation of the amino-acids. In the second, one must first submit

the material to hydrolysis in order to set free the amino-acids, after which one proceeds as before. There are various methods of isolating the amino-acids: one, by conversion into esters which are separated by fractional distillation; another, by extraction with butyl alcohol and subsequent separation; and for the separation of individual substances from a mixture, by the formation of insoluble derivatives. The general method is to practice esterification, and then after separation to distinguish the several amino-acids by means of some particular salt or derivative. The methods to be first described originated with E. Fischer.

The hydrolysis may be accomplished by either acids, alkalies or proteolytic enzyme. Usually the use of mineral acid is in every way to be preferred. But if tryptophane is to be sought for, the hydrolysis must be effected with trypsin. If the material is abundant, it is best to carry through a special search for tryptophane, and this will be described under that heading.

The material to be hydrolysed is suspended in hydrochloric acid of sp. gr. 1.19, three parts of the acid to one of the material to be analysed being used. The acid is then heated until the material has passed into solution, additional acid being added if needed. The solution is then boiled for some hours, under a reflux condenser. After the solution has cooled, insoluble humin is filtered off, and the precipitate washed until the wash water is clear. The clear solution is then evaporated to a small volume on a water-bath under diminished pressure, after which it is placed on ice for several days in order to give the hydrochloride of glutamic acid opportunity to crystallise out. If such crystallisation occurs, the crystals are removed by filtration and washed with dilute hydrochloric acid and further treated as described under that heading. The combined filtrates are evaporated to a small volume on the water-bath, under diminished pressure, to remove as much hydrochloric acid as possible. The residue is taken up in water and the process repeated. The residue is then diluted with water, heated on the water-bath, animal charcoal added, the solution filtered and the precipitate washed with hot water, the combined filtrates cooled and diluted to a known volume. In a small amount of this solution, an estimation of chlorine is then made, and exactly the necessary amount of sodium hydroxide added to neutralise the hydrochloric acid. This solution is then evaporated to a small volume under diminished pressure,

and placed on ice for several days to permit the tyrosine and cystine to crystallise out.

The crystals of tyrosine and cystine are collected and washed with ice cold water. They are then dissolved in hot dilute ammonia, cooled and neutralised with acetic acid. The tyrosine is quickly precipitated, though not quantitatively. It is collected, washed with ice water and further treated as described under tyrosine. The filtrate is then made ammoniacal and calcium chloride added to precipitate any phosphoric or oxalic acid that may be present. The filtrate is mixed with an equal volume of acetone, and the mixture acidified with acetic acid, following which the cystine will separate out, to be further treated as described under cystine.

The filtrate, after the separation of the tyrosine and cystine, is evaporated to a small volume under diminished pressure, and the residue taken up in absolute alcohol and again evaporated to a syrup, care being taken not to continue the process after bubbles form in the syrup. Three or four volumes of absolute alcohol are then added (care must be taken that a large flask is selected) and gaseous hydrogen chloride passed into the solution until it is saturated. Care must be taken that the gas is absolutely dry. Heat is applied during the process; finally the passage of gas is continued until the cooled alcoholic solution is saturated. In this process the amino-acids have been esterified and all pass into solution.

This alcoholic solution is now concentrated under diminished pressure, at a temperature of not over 40° , to about one-third or one-fourth of its volume, and placed on ice for 1 or 2 days to permit the hydrochloride of the ester of glycocoll to crystallise out; this is much facilitated by inoculation with a crystal of the substance. Care must be taken during these operations to prevent absorption of water by the alcohol. The crystallisation of the salt of the ester of glycocoll is then collected by filtration, washed with cold absolute alcohol and treated further as described under glycocoll.

The alcoholic filtrate is now evaporated to dryness at 40° under diminished pressure, absolute alcohol again added as before, and the process of esterification repeated by another introduction of gaseous hydrogen chloride. The solution is again treated exactly as before, to secure the separation of any further glycocoll, the solution being more concentrated before being placed on ice. The filtrate may then be submitted to the process for the separation of

the esters, or the process of esterification may be again repeated, and even again, if the attempt is being made to secure as nearly as possible the complete yield.

The final filtrate is evaporated to dryness at 40° , and the residue taken up in about 3 volumes of absolute alcohol, filtered and the chlorine content determined in a small fraction. The calculated needed amount of metallic sodium (excess to be carefully avoided) is then added to the amount of absolute alcohol necessary to make a 3% solution, and the cooled solution of sodium ethoxide added to the solution of esters. A little ether is then added and the mass placed on ice over-night. The sodium chloride is precipitated and should be filtered off.

(A different method of preparing esters of amino-acids, which avoids some of the losses occurring in aqueous solution, has been described (F. W. Foreman, *Biochem. J.*, 1919, **13**, 378). The amino-acids are converted into their lead salts, and these are suspended in alcohol and esterified by saturating with hydrochloric acid. The free hydrochloric acid and alcohol are then removed, the ester hydrochlorides dissolved in dry chloroform, and the esters liberated by shaking with anhydrous barium hydroxide.)

This solution is now subjected to fractional distillation. It is first distilled at 40° at 10 mm. pressure until all the alcohol-ether has passed over. The esters of glycocoll are not completely removed by crystallisation, and *d*-alanine distils over in this fraction. The distillate is mixed with dilute hydrochloric acid, the separated ether removed, and the acid solution, which now contains the hydrochlorides of the amino-acids mentioned, evaporated to dryness. Polariscopic examination of the aqueous solution of this residue will fix the presence of *d*-alanine. If it is present, the residue is to be esterified just as before, and the hydrochloride of the ester of glycocoll removed by crystallisation as before. The filtrate will contain fairly pure *d*-alanine; the ester is hydrolysed by boiling in 10 parts of water, and the *d*-alanine further treated as described under that heading.

A new receiver having been attached, the distillation of the mixture of esters is continued, the heat being slowly raised to 60° , with the pressure maintained at 10 mm. When nothing further comes over, another new receiver is attached and the heat raised slowly to 100° . The distillates are cooled with ice, and the distilla-

tion continued at 100° so long as there is a yield. The distilling flask is now transferred to an oil-bath, the pressure reduced to less than 1 mm., and the distillation continued at 100° so long as there is a yield. Finally, with a fourth receiver, with the pressure maintained below 1 mm., the distillation is continued with the temperature gradually raised to 180° , in fractions of about 20° each, until there is no further yield. There are then, therefore, 4 distillation fractions. In I, II and III may be found mixtures of proline, *d*-alanine, *d*-valine, *l*-leucine (possibly isoleucine) and such traces of glycocoll as may have escaped previous removal. In fraction IV may be found the esters of serine, phenylalanine, aspartic acid and such glutamic acid as may have escaped the previous crystallisation. While some leucine will be in all the first three fractions, most of it will be in the second and third, with the valine and possibly isoleucine. In the first fraction will be found most of the alanine with the glycocoll. Proline is to be recovered from all three.

The first three fractions are to be treated alike, though separately. The distillates are suspended in 10 parts of water and boiled under a reflux condenser to complete saponification, until the alkaline reaction disappears, this being accomplished in from 6 to 10 hours. The solution is then evaporated at low temperature and low pressure to a small volume and allowed to remain over night for a possible crystallisation of leucine. If such occurs, the crystals should be collected, and added to those later to be secured. Then the evaporation is continued to dryness. The dried residue is next extracted with boiling absolute alcohol, in which the proline is soluble, the others not to any great extent. These extractions are thrice repeated and the collected alcoholic extracts set aside to cool. On the following day the extract is filtered, and the residue extracted repeatedly with boiling absolute alcohol. After cooling, the final residue is returned to the original residue of each fraction. The final alcoholic extracts of the three fractions are now joined and evaporated to dryness. When taken up again in absolute alcohol, any further insoluble residue must be rejected. This alcoholic extract of proline is evaporated to dryness and further treated as described under that heading.

The residues of the three fractions are now dissolved in hot water, filtered through animal charcoal, concentrated to small

volume and set aside for crystallisation. The first crystallisation will be largely leucine, especially in fractions II and III. The filtrates are further concentrated and set aside for crystallisation; and this is repeated the third time, to the almost complete driving off of the water of solution. The first crystallisations are usually largely leucine, and to these should be added the previously secured yield of leucine. The second and third crystallisations of fraction I will consist largely of alanine and glycocoll, with a little leucine. The second and third crystallisations of fractions II and III will consist largely of valine and leucine, with possibly some isoleucine. There is, however, overlapping in all the crystallisations. *d*-Valine is soluble in methyl alcohol, and may be often secured in this manner, especially in the absence of isoleucine. Glycocoll may be secured by repetition of the esterification and crystallisation of the hydrochloride of the ester. The different fractions of crystals are then to be tested for the several compounds and derivatives which will be described under the different headings.

The distillate contained in fraction IV is taken up in 5 volumes of water and shaken with ether in a separating funnel until the aqueous solution is clear, the ester of phenylalanine passing into the ether. The ethereal extract is then separated and washed several times with water, the washings being returned to the original aqueous solution. The ether is evaporated to dryness, the residue taken up in dilute hydrochloric acid and again evaporated to dryness, in which process saponification occurs. The hydrochloride may then be secured by crystallisation, and subjected to the tests for the substance to be described under that heading.

The aqueous solution is saponified by boiling with barium hydroxide, using about twice the amount of the weight of the distillate. The mixture is boiled for 2 hours under a reflux condenser, and then set aside over-night. The barium salt of racemic aspartic acid separates out, is collected on a filter, and washed with barium hydroxide solution. The salt is then suspended in 25% sulphuric acid, boiled, filtered, and the excess of sulphuric acid removed by the careful addition of barium hydroxide, and the liquid again filtered. From the filtrate aspartic acid may be recovered by crystallisation. This is to be added to the fraction later to be secured and treated further as will be described under that heading.

The filtrate remaining after removal of the barium salt of aspartic acid is freed from barium with sulphuric acid, evaporated to dryness under diminished pressure, the residue dissolved in hot water, filtered through animal charcoal, and the solution saturated with gaseous hydrochloric acid. On standing, the hydrochloride of glutamic acid crystallises out. This is collected, joined to the fraction that was early obtained, recrystallised from dilute hydrochloric acid, and identified as described under that heading.

The filtrate contains the rest of the aspartic acid and serine. It is evaporated to dryness under diminished pressure, and, for the better removal of the hydrochloric acid, the residue is again taken up in water, and the liquid again evaporated to dryness. This residue is taken up in water, the chlorine removed by the controlled addition of yellow lead oxide with boiling, the liquid filtered, the excess of lead removed with hydrogen sulphide, and the filtrate concentrated, following which the aspartic acid will separate out. The filtrate contains the serine in the racemic state, and on careful neutralisation and further concentration much of the serine will crystallise out. The serine is then further to be tested by the methods described under that heading.

The methods are not quantitative. Loss occurs at each operation—esterification, distillation, and saponification. Some evidence has been obtained as to the percentage loss under the best conditions of alkali, etc., by experienced workers, starting with pure amino-acids. The percentage yields obtained after carrying through the three operations were as follows: glycocoll 65%; *d*-alanine 64%; *l*-leucine 75% (in another case 88.8%); *d*-valine 68%; *l*-phenylalanine 54%; *l*-proline 69.5%; aspartic acid, about 60%; glutamic acid, about 70% (in another case 85%). In the hydrolysed protein material, which is separated by fractional distillation of the esters, it has been suggested that the fraction which formerly was distilled above 110° at less than 1 mm. pressure should be worked up directly without distillation, this fraction being dealt with as easily in this way as by the older method.

When investigating a material containing pre-formed amino-acids, the material should be evaporated to dryness, extracted with hot hydrochloric acid, sp. gr. 1.15, filtered and then set aside for a possible crystallisation of glutamic acid. Following this, the material is treated exactly as described. This is the best method

for the analysis of urine; the direct precipitation of the amino-acids, as sometimes advised, by β -naphthalene-sulpho-chloride is liable to fail, even if the amino-acids are present in abundance.

Isolation of Diamino-acids

For the isolation of histidine and the diamino-acids, arginine and lysine, it is better to use a separate lot of the material. The material is extracted in hot acidified water, if one is to search for the pre-formed substances. If the material is a protein, it is to be hydrolysed as previously described; sulphuric acid of 25% strength is preferable, and the acid removed by precipitation as barium sulphate, filtered and washed five times with hot water. The material is now to be treated alike in each instance. Sulphuric acid is added to make a concentration of 5%, and a solution of 10% phosphotungstic acid in 5% sulphuric acid is added so long as a precipitate forms. This precipitates histidine, arginine and lysine; but the amino-acids are not precipitated from dilute solution. The precipitate is washed with dilute sulphuric acid, suspended in water, the metal precipitated by the addition of barium hydroxide until the reaction is alkaline, filtered, and the precipitate thoroughly washed, the excess of barium removed from the filtrate by the addition of sulphuric acid to the point of distinct acidity, and again filtered and the precipitate washed. The filtrate is then transferred to a large flask and a boiling hot solution of silver sulphate added, with vigorous shaking, until a drop of the supernatant liquor gives a brownish reaction when dropped into a watch-glass containing a solution of barium hydroxide. The mixture is cooled and saturated with powdered barium hydroxide, so much being used that an excess lies with the reaction-precipitate in the bottom of the flask. This is collected on a Buchner funnel, the precipitate rubbed up in a mortar with purified sand and a saturated solution of barium hydroxide, again collected on the Buchner funnel, thoroughly washed with barium hydroxide, and the filtrates and wash waters collected. The filtrate contains the lysine; the precipitate, the arginine and histidine.

The precipitate is suspended in water and thoroughly mixed, sulphuric acid being added to acid reaction. Hydrogen sulphide is then passed in to precipitate the silver, and when this is completed the mixture is heated to steaming and filtered hot, the pre-

cipitate extracted and washed with hot water repeatedly, until the washings no longer react with phosphotungstic acid, and the combined filtrates concentrated to a known volume. A Kjeldahl estimation of nitrogen is then made. The solution is carefully neutralised with barium hydroxide solution and then a solution of barium nitrate added, drop by drop, so long as a precipitate forms. This precipitate of barium sulphate is filtered off and washed, and the filtrate concentrated if necessary. It is next acidified with nitric acid, and silver nitrate solution added until a drop of the supernatant fluid produces a brownish reaction with barium hydroxide solution. Then the solution is carefully neutralised with barium hydroxide solution, a little suspended barium carbonate added, the mixture heated to boiling, and set aside to cool. Histidine is precipitated as the silver compound; the silver compound of arginine remains in solution at neutral reaction. The precipitate is collected on a filter and washed with very dilute baryta water until the wash water is free from nitric acid, and the filtrates collected. The precipitate is heated in water, and sulphuric acid added to the point of an acid reaction, the silver precipitated with hydrogen sulphide, filtered off, and washed thoroughly, the collected filtrates concentrated, and the histidine content estimated by a nitrogen determination. The histidine in the solution must now be further treated as described under that heading.

The filtrate containing the soluble silver salt of arginine is saturated with barium hydroxide, which precipitates the arginine compound. The precipitate is collected on a Buchner funnel, thoroughly extracted and washed, after which it is suspended in water, acidified with sulphuric acid, and the silver precipitated with hydrogen sulphide, the precipitate removed by hot filtration and washed, the collected filtrates concentrated, and the sulphuric acid removed by barium hydroxide. The excess of barium is removed by means of carbon dioxide, and the filtrate concentrated and submitted to a Kjeldahl estimation for nitrogen, from which the amount of arginine is calculated. The solution of arginine is then to be treated as described under that heading.

The filtrate containing the lysine is acidified with sulphuric acid, freed from silver by hydrogen sulphide and filtered hot, and the collected filtrates concentrated. Sulphuric acid is added to make the concentration about 5%, and the lysine precipitated by the

addition of phosphotungstic acid. After 24 hours the precipitate is collected on a filter, washed with dilute sulphuric acid, and the precipitate suspended in hot water, barium hydroxide added until the reaction is alkaline, the insoluble salt of tungsten removed by filtration and washed, and the collected filtrates freed from barium by means of carbon dioxide, and concentrated almost to dryness. The residue is taken up in hot water, filtered, and a nitrogen estimation of the filtrate made, from which the lysine may be calculated. The solution of lysine should then be further treated as described under lysine.

The group reactions of the amino-acids, by means of which we obtain derivatives upon which we rely for the identification of the different amino-acids, are carried out as follows:—The different amino-acids should be isolated as far as possible, as previously described, since all these derivatives form their characteristic compounds best when pure. The amino-acid solution is made alkaline with sodium hydroxide to about normal alkalinity. Then the solution is placed in a separating funnel, a fair amount of a 10% solution of pure β -naphthalene-sulphochloride in ether added, and the funnel shaken in a machine. Every hour the reaction of the aqueous solution is tested, and alkali added if necessary to maintain decided alkalinity. After shaking for 10 hours, the aqueous solution, which contains the derivative, is separated and filtered. It is then acidified and extracted thoroughly with ether, which takes up the derivative. The ethereal extract is washed with water to remove the acid reaction, and the ether evaporated to dryness. The residue is taken up in hot dilute ammonia, filtered through animal charcoal, the filtrate acidified and again extracted with ether, the ethereal extract washed with cold water and evaporated to dryness. This residue is taken up in hot dilute ammonia, acidified with hydrochloric acid, and the compound permitted to crystallise out. Fractional crystallisation and subsequent purification may be necessary.

To the amino-acid, dissolved in normal alkali, is added in small portions, an amount of phenyl-iso-cyanate equal to that of the amino-acid, the mixture being kept cool and vigorously shaken for an hour. Animal charcoal is then added, and the mixture filtered, following which the filtrate is acidified and set on ice. The next day the precipitate is collected, taken up in 4% hydrochloric acid, and concentrated on the water-bath, later to be set aside for crystal-

lisation. With some amino-acids the phenyl-iso-cyanate derivative crystallises well; with others the corresponding hydantoin is to be preferred.

The derivatives of α -naphthyl-iso-cyanate are obtained in the same manner, being soluble in alkali and insoluble in acid solution. They may be recrystallised well from hot alcohol.

To form the derivatives with benzoyl chloride, the solution of amino-acid is made alkaline with sodium carbonate and benzoyl chloride added, with vigorous shaking, so long as it is taken up. After a few hours the mixture is filtered, acidified with hydrochloric acid, and set aside in a cool place for crystallisation. The precipitate is collected, washed in cold acidified water, dried, and the benzoic acid extracted with petroleum spirit. The remaining salt may then be redissolved in alkaline water, and recrystallised from the acidified medium.

To form the carbamino-acid derivative, the solution of the amino-acid is saturated with carbon dioxide, chilled, and lime water added, following which carbon dioxide is continuously added until the liquid is saturated. Then lime water and crystallised calcium carbonate are added, and the mixture vigorously shaken. After filtration, chilled alcohol is added to the filtrate until opacity appears. On standing the calcium salt of the carbamino-acid crystallises out.

A method of separation of the products of hydrolysis of a protein by extraction with butyl alcohol and dispensing with the esterification and distillation procedures, has been developed (H. D. Dakin, *Biochem. J.*, 1918, **12**, 290) and should prove to be a most valuable and useful method. Butyl alcohol is obtainable at comparatively low cost, because of its large production in the fermentation process of acetone manufacture. The essential features of this new separation may be given in Dakin's own words.

On extracting amino-acid solutions from caseinogen and other proteins with butyl alcohol, it was found that the proline and practically the whole of the monamino-acids were extracted, together with some peptide anhydrides. The strongly ionised dibasic and dicarboxylic acids were left behind practically quantitatively. The only "neutral" monamino-acids detected in the extracted material have been minute traces of serine, and in the case of amino-acids from gelatin the complete extraction of glycine seemed more

difficult than that of the other amino-acids. In general, the extraction is surprisingly rapid, and with the simple apparatus employed the extraction of two or three hundred grm. of mixed amino-acids is complete in 36 hours. The bulk of the monamino-acids separate out in the extraction flask as a cream-coloured granular powder. It is convenient to filter off the monamino-acids occasionally during the extraction in order to avoid bumping or local over-heating, using the mother liquor for continuing the extraction. The solid monamino-acids are washed first with a little butyl alcohol, then with a little ether, which removes traces of pigment and water. The butyl alcohol mother liquor contains the whole of the proline, together with small amounts of amino-acids and peptide anhydrides, which can be largely separated through their sparing solubility in alcohol or water.

On examining the residue of non-extracted amino-acids, it was found to contain practically the whole of the diamino-acids, as judged by quantitative precipitation with phosphotungstic acid, and also the dibasic acids, aspartic acid, glutamic acid, and a new dibasic acid. Larger yields of the dibasic acids were obtained from caseinogen than have previously been obtained by other methods. None of the dibasic acids could be detected among the extracted monamino-acids, so that it is clear that, for practical purposes, they may be regarded as quantitatively separated from the latter.

It is thus seen, that by the use of butyl alcohol as a solvent, the products of hydrolysis of a protein may be readily separated almost completely into the following five groups:

(1) Monamino-acids, both aliphatic and aromatic, insoluble in alcohol but extracted by butyl alcohol.

(2) Proline, soluble in alcohol and extracted by butyl alcohol.

(3) Peptide anhydrides (diketopiperazines) extracted by butyl alcohol, but separated from (2) by sparing solubility in alcohol or water.

(4) Dicarboxylic acids, not extracted by butyl alcohol.

(5) Diamino-acids, not extracted by butyl alcohol, but separable from (4) by phosphotungstic acid and other means.

No indications of any racemisation were observed. All the amino-acids separated possessed their full optical rotation, thus limiting the number of isomers in the various mixtures and permitting greater use of the polarimeter for identification purposes. The

absence of racemisation has an obvious bearing on the use of the method for purposes of preparing material for bacterial and animal metabolism experiments. Materially higher yields of many amino-acids may be obtained by this method than by existing methods, thus permitting a more nearly quantitative analysis of the proteins themselves.

The carrying out of the method with caseinogen as example will be described.

Hydrolysis.—Purified caseinogen varying in amounts from 100–400 grm. was hydrolysed by boiling for 12 to 16 hours, with five to six times its weight of dilute sulphuric acid prepared by mixing the concentrated acid with three volumes of water. Hydrolysis under these conditions is still incomplete, judged by the subsequent separation of peptide anhydrides in significant amount. After the heating was ended, the solution was diluted with water and the sulphuric acid quantitatively removed with barium hydroxide in the usual way. The filtrate, which still reacts acid to litmus but not to Congo-red, was concentrated moderately, and about 2% of crude tyrosine allowed to crystallise out. The filtrate was again concentrated and made approximately neutral to litmus by the further addition of barium hydroxide. The optimum reaction has still to be determined and probably varies with different proteins. In the case of gelatin feebly acid and alkaline reactions obtained by adding acetic acid or ammonia were successively tried, but this is not necessary with caseinogen, and it is doubtful if it is ever desirable.

Extraction.—The approximately neutral solution of amino-acids, prepared as above described, was concentrated on the water-bath until crude leucine began to separate and then the whole mixture was transferred while still warm to the extraction apparatus. Usually an apparatus of the simple Kutscher-Steudel type was employed, although others proved equally satisfactory. The amino-acids from 100 grm. of caseinogen may be conveniently extracted in an apparatus holding about 350 c.c. It is preferable to arrange conditions so that the aqueous layer occupies three-quarters to five-sixths of the available space and the volume of supernatant butyl alcohol is relatively small. It has been found convenient to substitute a rather larger extraction flask of the Jena type for the smaller flask usually employed. Rubber connections can be employed, although they are attacked by the hot alcohol to a

considerable extent, and their exposed surface should be reduced to a minimum. The extraction flask is heated over a sand-bath, preferably with rather high sides, so as to reduce condensation in the flask, and the flame is adjusted so that a reasonably rapid flow of alcohol returns into the flask. When using a 300-400 c.c. or smaller apparatus, the aqueous solution soon attains a temperature of from 60° to 80°, and neither cooling nor heating is required, but when larger volumes of fluid are being extracted, it is a good plan to warm it to 75° or 80° by means of a water-bath.

Soon after the extraction has begun, a separation of cream-coloured granular particles of amino-acids is observed in the extraction flask, and the amount steadily increases. After extraction has continued during a working day, it will be found convenient to allow the extract to stand over-night and to filter off the separated amino-acids on a Buchner funnel, washing them with a little butyl alcohol, and finally with a little ether. The combined alcoholic filtrates, which are light brown in colour, are used for continuing the extraction on the second day. Risk of breakage due to the accumulation of solid amino-acids on the bottom of the flask is thus avoided, and it is possible to judge the completeness of the extraction by observing the increment of solid amino-acids on further heating. When, after several hours' extraction, no significant additional amount of solid matter separates from the butyl alcohol solution, the operation is regarded as finished. With the apparatus employed and extracting 100 to 400 grm. of mixed amino-acids, the operation usually took about 36 hours, the time varying naturally with the rate at which the butyl alcohol is boiled.

When the extraction is over, the aqueous solution is reserved for examination for bases and dicarboxylic acids, as will be described later. The butyl alcohol extract, containing the proline and some peptide anhydrides, is filtered from the separated amino-acids, after standing at room temperature for some hours, while the monamino-acids, filtered and washed as previously described, are dried and weighed.

Butyl Alcohol Extract. Separation of Proline, Etc.—The filtered extract is evaporated to dryness in a distillation flask under reduced pressure, and the sticky brownish residue boiled with about ten parts of absolute alcohol. In the case of caseinogen, at least, the whole of the residue is soluble in ethyl alcohol, indicating the absence

of any considerable quantity of monamino-acids other than proline. On allowing the alcoholic solution to stand over-night, crystallisation occurs, with separation of a small quantity, averaging 2% of the caseinogen, of an impure mixture chiefly composed of peptide anhydrides with traces of monamino-acids. This product is filtered off, washed with alcohol and examined later. The alcoholic filtrate is then evaporated under reduced pressure, dissolved in hot water, boiled with charcoal, filtered and concentrated to syrup.

A further crystallisation of peptide anhydrides occurs on standing, averaging 2 to 3% of the caseinogen taken, and this is filtered off and examined, together with the similar product obtained above. The total yield of peptide anhydrides from caseinogen in different experiments ranged from 2.2 to 5.7%, the larger yields being obtained in experiments with the larger amounts of the protein.

The clear aqueous solution, after separation of the two fractions just described, contains the whole of the proline. It is already much purer than might be anticipated from its method of preparation, and compares favourably with the alcohol-extracted product obtained by the ester method. There is no evidence of any racemisation of the proline as obtained by the direct extraction method, as judged by the solubility of copper salts in methyl alcohol and by the precipitation of other derivatives, but, on the other hand, the impurities which accompany the proline appear to be fairly strongly dextro-rotatory, so that a direct polarimetric estimation of proline is not possible. The observed rotations correspond to about three-fourths of the proline believed to be actually present. The actual content of proline in caseinogen, as estimated by van Slyke's methods for differentiating between amino and imino nitrogen, and by the preparation of *l*-prolylhydantoin, was found to average 8.0 %, a value higher than that obtained by the use of other methods.

Monamino-acids Extracted by Butyl Alcohol.—The amino-acids extracted by butyl alcohol other than proline, separate, as previously described, as a cream-coloured granular deposit. They dissolve in water to give a clear light yellow solution and contain only insignificant traces or none of the diamino-acids or dibasic acids. Including about 2–3% of tyrosine filtered off prior to extraction, the total yield is approximately 40% of the caseinogen taken, and is made up chiefly of alanine, valine, leucines, and phenyl-

alanine, with a little tyrosine. The product is in a most convenient form for application of the ester method.

Amino-acids Not Extracted by Butyl Alcohol. Separation of Aspartic, Glutamic, and Other Acids.—As has already been stated, the amino-acids which are not extracted by butyl alcohol from aqueous solution, are the strongly ionised diamino-acids and dicarboxylic acids. Quantitative experiments, in which the mixture was precipitated with phosphotungstic acid, showed that the whole of the diamino-acids could be recovered in this way, but the filtrate yielded less of the dicarboxylic acids than could be obtained by other methods, so that for purposes of quantitative analysis of proteins, it is decidedly preferable to use an aliquot portion for the examination of the bases and the rest for the dicarboxylic acids.

The Diamino-acids.—The precipitation with phosphotungstic acid, and subsequent separation of histidine, arginine and lysine, requires no comment other than the desirability of removing dissolved butyl alcohol from the solution by heating before precipitation, for the inhibitory action of alcohols on the precipitation is well known.

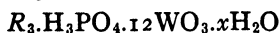
The Dicarboxylic-acids.—The degree of purification effected by the removal of the mono-amino-acids by extraction, permits of a much more nearly quantitative separation of glutamic acid as hydrochloride than is usually the case. Precipitation, moreover, is usually more rapid, and after removing the first crop of crystals, concentrating and re-saturating with hydrochloric acid in the usual way, the second crop of crystals is very small. It may be noted here that, in the analysis of gelatin and other proteins containing much glycine, the precipitation of glycine hydrochloride along with the glutamic acid hydrochloride is possible on long standing in concentrated solution. An average of glutamic acid of 21% was found by this method in caseinogen.

To separate aspartic acid, the filtrate from the glutamic acid hydrochloride is concentrated under reduced pressure to remove as much of the hydrochloric acid as possible, and the resulting syrup dissolved in about ten parts of hot water. It is then treated with calcium hydroxide and the calcium salts precipitated by alcohol (Foreman). The sticky deliquescent calcium salts, after washing with alcohol, are dissolved in water, and the calcium removed exactly with oxalic acid. Freshly precipitated and well-washed lead

hydroxide is then added by degrees, while the solution is kept boiling under a reflux condenser. The addition of lead hydroxide is continued until the reaction of the solution is distinctly alkaline to litmus, and heating is then continued for a further 15 minutes, after which the mixture is allowed to cool and stand over-night. The precipitate containing the aspartic acid and much lead chloride is filtered off and washed with cold water. The lead precipitate is somewhat difficult to decompose with hydrogen sulphide, so that it is more convenient to boil it with dilute sulphuric acid in moderate excess. Almost all the lead is thus removed, and the filtrate contains aspartic, hydrochloric, and sulphuric acids. The filtrate is now concentrated with excess of precipitated barium carbonate, filtered and made up to a convenient volume, such as 1 c.c. per grm. of protein taken. A nitrogen estimation on a small aliquot part gives an indication of the possible aspartic acid concentration. A hot concentrated solution of normal copper acetate is then added in amount equal to 1.5 mols. per mol. of aspartic acid. On allowing the solution to stand for a couple of days in a cool place, the characteristic copper aspartate separates out almost completely and is filtered off, washed, dried and weighed. The salt, after drying in air, contains very nearly 3 mols. of water; dried at 130° it is anhydrous. 3.5 and 4.1% of aspartic acid were found in caseinogen in two determinations.

The separation of pairs of amino-acids from each other will be dealt with in connection with the description of the individual substances.

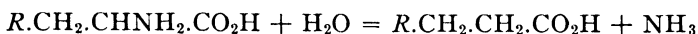
Phosphotungstic acid is being widely used as a precipitant for basic substances. A careful study (J. C. Drummond, *Biochem. J.*, 1918, 12, 5) has shown that for precipitating amino-acids and other basic bodies from their solutions, the most favourable conditions include a slight excess of the precipitant in 5% sulphuric acid solution. Several of the precipitates are soluble in excess of the precipitant. To carry out the precipitation, a 30% solution of phosphotungstic acid in 5% sulphuric acid is added to the base dissolved in sulphuric acid, allowed to stand 24 hours, filtered, washed with 5% sulphuric acid, and recrystallised from water or 50% ethyl alcohol. Well-defined crystalline products are obtained in this way, having the general formula—



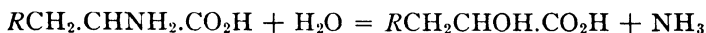
The general reactions involved in the bacterial decomposition of amino-acids, as possible examples of such actions with proteins, may be outlined here before going on with the description of the individual amino-acids.

These bacterial decompositions of amino-acids form one step in the chemical changes through which proteins are transformed by bacteria into simpler compounds incidental to their utilisation for energy.¹ Each kind of organism utilises proteins or protein derivatives somewhat differently and characteristically, but, in general, one or more of the following reactions are involved.

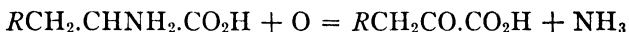
- (1) Reduction and decomposition of amino-acid to fatty acid.



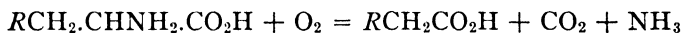
- (2) Hydrolytic deamination of amino-acid to hydroxy-acid.



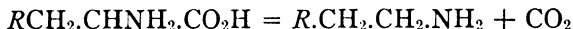
- (3) Oxidation and deamination of amino-acid to alpha-ketonic acid.



- (4) Deamination and oxidation of amino-acid to fatty acid with one less carbon atom.



- (5) Decarboxylation of amino-acid with formation of amine.

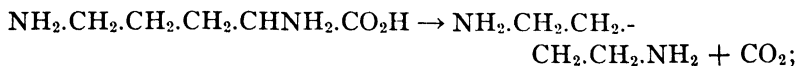


Tyrosine, by the action of bacterial organisms such as *Bacillus proteus*, forms paracresol and phenol, which have no available energy for the organism, and are therefore eliminated from the bacterial cell and appear in the culture medium, or in the alimentary canal.

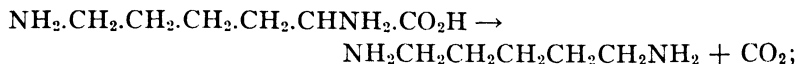
Tryptophane, by the action of bacterial organisms decomposes, with the formation of a number of products. Indol may be formed in the intestinal tract by *Bacillus coli* and by *Bacillus proteus*, especially where intestinal putrefaction is actively taking place. Indol acetic acid is formed by oxidative deamination of tryptophane. Skatol is also a common product.

¹ Cf. especially A. I. Kendall, Chapter on "The Chemistry of Bacterial Metabolism" in "Endocrinology and Metabolism," D. Appleton and Company, New York and London, 1922, Volume 3, pp. 678-689.

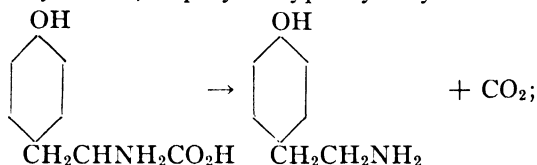
Ornithine is changed by mixtures of bacteria acting upon protein into putrescine or tetramethylenediamine



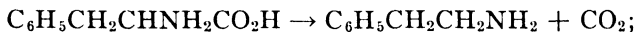
lysine similarly into cadaverine or pentamethylenediamine



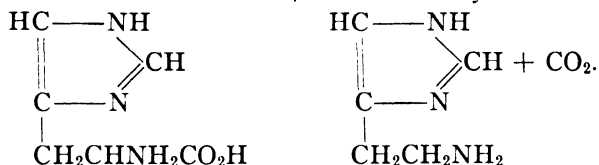
tyrosine into tyramine, or *p*-hydroxyphenylethylamine



phenylalanine into phenylethylamine

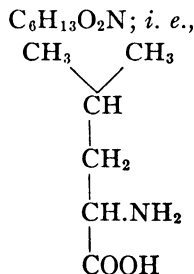


and histidine into histamine or β -imidazole-ethyl-amine



These changes are chiefly the result of the activities of the colon-proteus-mucosus-capsulatus group of bacilli.

Leucine (α -Amino-iso-butylacetic Acid)



Leucine was originally discovered by Proust (1819) in putrefied cheese. It has been found in the brain, pancreas, thyroid and thymus glands, etc.; and in the liver and urine in cases of smallpox, typhus fever, leucæmia, affections of the spinal cord, sepsis, acute

yellow atrophy, and poisoning by phosphorus. Leucine is a characteristic product of the putrefaction of proteins. It is produced, together with the other amino-acids, aspartic and glutamic acids, by the action of boiling dilute acids or fused potassium hydroxide on these substances.

In the vegetable kingdom, leucine has been found in young pumpkins, beetroot and beetroot molasses, the juice of vetches germinated in the dark; in *Agaricus muscarius*, etc., etc.

Leucine has been prepared synthetically by the reaction of α -bromohexioic acid with ammonia.

Leucine is conveniently prepared by boiling 2 parts of horn shavings with 5 parts of sulphuric acid and 13 of water for 24 hours, under a reflux condenser. The product is treated with excess of lime, boiled well, filtered, and the filtrate evaporated to about one-half. It is then faintly acidified with oxalic acid, the calcium oxalate filtered,¹ and the filtrate concentrated till a crystalline film forms on the surface. On cooling, crystalline laminæ are deposited, consisting of a mixture of leucine and tyrosine, and a further crop can be obtained by concentrating the mother liquor. The crystals are redissolved in such a quantity of boiling water that only tyrosine is deposited on cooling. The mother-liquor is treated with precipitated lead hydroxide, which removes colouring matter and a little tyrosine, and the filtrate freed from lead by hydrogen sulphide and evaporated till a film forms on the surface. The crystals of leucine which deposit on cooling may be further purified from tyrosine by treatment with boiling alcohol of 70% (sp. gr., 0.872), which leaves the tyrosine undissolved. To get rid of traces of a sulphuretted impurity, the leucine may be dissolved in dilute alkali hydroxide, a solution of lead oxide in caustic alkali added, and the liquid boiled for half an hour. The liquid is filtered from the lead sulphide, exactly neutralised with sulphuric acid, evaporated to complete dryness, and the residue exhausted with boiling alcohol of 0.830 sp. gr., which, on cooling, deposits the leucine in a state of absolute purity. The crystals melt, with decomposition, at 293–295° if heated rapidly in a closed capillary tube.

Leucine forms colourless thin 6-sided plates and narrow rod-like plates (Fig. 2). The crystals have a sp. gr. of 1.293, but are wetted with difficulty by water, and often float on the surface. When not

¹ At this stage Waage gradually adds recently precipitated cupric hydroxide (avoiding excess) and boils. On cooling, a copper compound of leucine separates out in light blue scales. This is filtered off, decomposed by hydrogen sulphide, and the liberated leucine purified from dilute alcohol by crystallisation.

perfectly pure, leucine often separates in concentric nodules closely resembling fatty cells (Fig. 3), but which under the microscope appear as concentrically grouped, highly refracting needles.

When cautiously heated (in a tube open at both ends), leucine sublimes unchanged in light white flocks, which under the microscope are seen to consist of delicate scales grouped together in the form

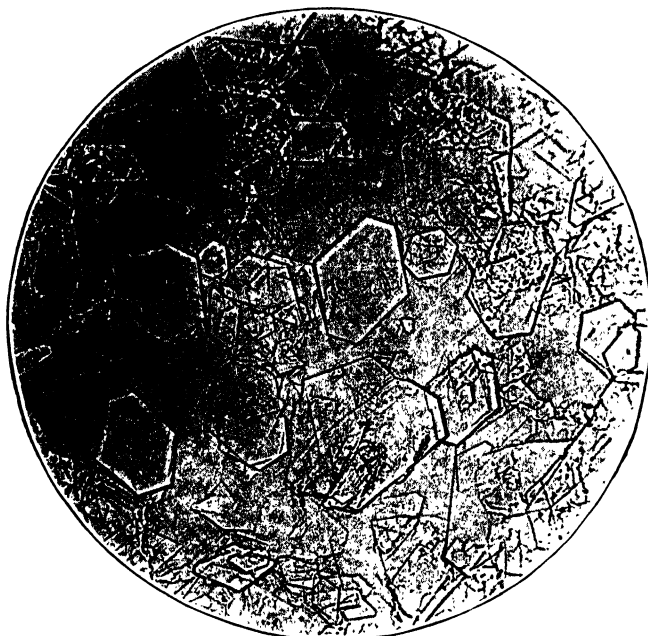


FIG. 2.—Leucine. $\times 100$. (From Keenan, *J. Biol. Chem.*, 1924, **62**, 163.)

of rosettes. When heated to 170° , leucine melts to a brown viscous liquid, and at a slightly higher temperature decomposes into amylamine and carbon dioxide:



Leucine is sparingly soluble in cold water (1:40), but more readily in boiling water. It requires 660 parts of cold 50% alcohol and 1,040 parts of cold 96% alcohol for solution, but dissolves in 800 parts of boiling 98% alcohol, and more readily in weaker spirit. Its solubility in water and in alcohol is increased by acetic acid or an acetate of an alkali metal. Leucine is insoluble in ether. It dissolves readily in acids and alkalies.

Leucine, as obtained in the hydrolysis of proteins, is optically active; the *l*-leucine so isolated rotates the plane of polarised light to the left in aqueous solution ($[\alpha]_D^{20} = -10.4^\circ$), but to the right in hydrochloric acid solution, $[\alpha]_D^{20} = +15.6^\circ$.

When heated with barium hydroxide to 160° it racemises, and forms the inactive modification which has been proved to be identical with the α -amino-isobutyl-acetic acid prepared synthetically from isovaleraldehyde.

When fused with alkali hydroxide, leucine yields normal valeric acid ($C_5H_{10}O_2$), ammonia, hydrogen, and carbon dioxide. When

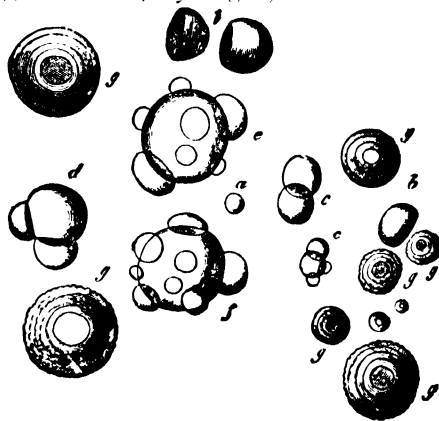


FIG. 3.—Spheroidal crystalline masses of leucine. *a*, a very minute simple spherule; *b*, hemispheroidal masses; *c*, aggregates of small globules; *d*, a large globule supporting two halves; *e*, *f*, a large spheroid of leucine richly studded with minute segments; *g*, *g*, *g*, laminated globules of leucine, some with smooth, some with rough surface, and of very various sizes.

heated with fuming hydriodic acid to 140° it yields caproic acid and ammonia, $C_6H_{13}ON_2 + H_2 = C_6H_{12}O_2 + NH_3$. Nitrous acid decomposes leucine into nitrogen and hydroxycaproic or leucic acid, $C_6H_{10}(OH).CO.OH$.

An aqueous solution of leucine is coloured a deep red by ferric chloride.

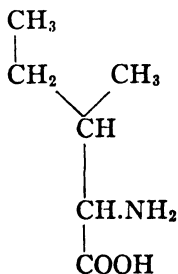
B_2HCl forms crystalline scales, very soluble in water. $B_2H_2PtCl_6$ forms yellow crystalline grains, soluble in water, but insoluble in alcohol. $B.HNO_3$ forms colourless needles, very readily soluble in water.

Copper amino-caproate, $Cu(C_6H_{12}O_2N)_2$, is obtained by adding recently precipitated cupric hydroxide to a strong aqueous solution

of leucine, and boiling the liquid. A bluish solution results, which, on cooling, deposits light blue scales, which require 3,045 parts of cold or 1,460 parts of boiling water for solution. This reaction may be employed for the isolation of leucine, but the solubility of the copper compound is materially increased by the presence of certain organic matters. With excess of copper oxide leucine forms an insoluble compound. $\text{Pb}(\text{C}_6\text{H}_{12}\text{O}_2\text{N})_2 + \text{H}_2\text{O}$ is deposited in mirror-like plates or nacreous scales, on cautiously adding ammonia to a boiling solution of leucine to which lead acetate has been added. In the isolation of leucine, as described under the ester method, several fractions of crystals of leucine are secured, partly mixed with valine and possibly with alanine and even glycocoll. Fractional crystallisation must be relied upon in the main, to secure the pure leucine. The hydrochloride of the ethyl ester melts at 134° . The ester forms also a picrate which melts at 128° . The most characteristic derivative of leucine is the α -naphthyl-iso-cyanate, long thin needles, melting at 163.5° . β -Naphthalene-sulpho-leucine crystallises in fine prisms which melt at 68° to an oily drop. The phenyl-iso-cyanate is to be recrystallised from warm alcohol by the addition of warm water, and forms flat leaves and prisms that melt at 165° with the formation of gas. The picrolonate melts above 150° (indefinite) and is soluble—0.55 part in 100 parts water at $20\text{--}23^\circ$.

Leucine may be identified by means of the microscopic appearance of its crystals, the hydrochloride and picrate of its ethyl ester, the phenyl- and α -naphthyl-iso-cyanate compounds, the β -naphthalene-sulpho compound, and the isobutylhydantoin, m. p. 205° , formed by boiling leucine with excess of urea and barium hydroxide.

Iso-leucine (α -Amino- β -methyl- β -ethyl-propionic acid)

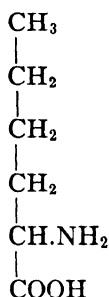
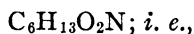


Iso-leucine was first isolated from molasses residues by F. Ehrlich. It is obtained in the hydrolysis of a number of proteins, frequently accompanying leucine, with which it forms mixed crystals. The largest amount obtained up to the present is 2.96% from a hetero-albumose from fibrin. Its structure was proved by the formation of *d*-amyl alcohol in the fermentation with sugar and yeast, and by its synthesis from *d*-amyl alcohol. It may also be separated from leucine in the products of protein hydrolysis by the greater solubility of its anhydrous copper salt in methyl alcohol.

Iso-leucine crystallises in leaflets or rods in the rhombic form. It does not melt up to 250°. It is more soluble in water (1:25.8) than leucine, and has a bitter taste. It is dextrorotatory; in water, $[\alpha]_D^{20} = +9.74^\circ$, in 20% HCl, $[\alpha]_D^{20} = +36.8^\circ$. Its copper salt is readily soluble in methyl alcohol.

The benzoyl compound melts at 116–117°; benzoyl-sulpho-isoleucine melts at 149–150°; the phenyl isocyanate compound melts at 119–120°; the naphthyl isocyanate compound melts at 178°; the naphthalene-sulpho derivative, recrystallised from ethyl alcohol melts at 200–203°; and *p*-toluene-sulpho-isoleucine melts at 226°. The picrolonate melts at 170°, and its solubility in water is 0.58 part in 100 at 20–23°.

Nor-leucine. (α -Aminocaproic acid)

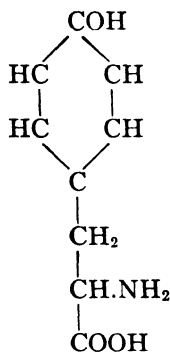


Nor-leucine was discovered by Abderhalden and Weil in the products of hydrolysis of the proteins of nerve tissue. It was synthesised from α -bromocaproic acid and ammonia, and the racemic mixture resolved into its optically active components by means of the brucine salt of its formyl derivative.

Nor-leucine crystallises from water in scale-like six-sided leaflets united to form nodules, which sinter at $275-280^{\circ}$ and melt at 301° , subliming to a great extent. It has a slightly sweet taste. It is soluble in water to some extent (1.5:100), insoluble in methyl and ethyl alcohols. It is dextrorotatory as obtained; in water, $[\alpha]_D^{20} = +6.53^{\circ}$. Its copper salt crystallises from water as dark blue needles.

Tyrosine (*p*-Hydroxyphenyl- α -aminopropionic acid)

$C_9H_{11}O_3N$; *i. e.*,



Tyrosine occurs ready-formed, and almost always accompanied by leucine, in both the animal and vegetable organisms. It is likewise produced, together with leucine, by the putrefactive decomposition of proteins, or by their treatment with alkalies or acids, and has also been obtained synthetically.

A method of preparing tyrosine is described on page 253. Another plan is to add dry casein, fibrin, or albumin (free from fat) gradually to an equal weight of fused or highly concentrated solution of potassium hydroxide contained in a capacious iron vessel. The heating, which is accompanied by an evolution of ammonia and most disagreeable odour, is continued until evolution of hydrogen commences and the fused mass changes in colour from brown to yellow. The product is then poured out, dissolved in hot water, and the solution slightly acidified with acetic acid. On cooling, an abundant crop of crystals of tyrosine separates in concentric groups of needles, which may be purified by re-solution in hot water containing potassium carbonate and precipitation with acetic acid.

For further purification, the tyrosine should be recrystallised from hot water containing acetic acid. For the removal of obstinately adhering cystine, Städeler adds to the warm aqueous solution a small quantity of basic lead acetate, treats the filtered liquid with hydrogen sulphide, and recovers the tyrosine by concentrating the filtrate to the crystallising point.



FIG. 4.—Tyrosine. $\times 120$. (From Keenan, *J. Biol. Chem.*, 1924, **62**, 163.)

Tyrosine is deposited from its hot aqueous solution in stellate groups of long, slender, silky needles, which on drying become felted together to a snow-white mass. From ammoniacal solutions it is deposited in larger needles, also having a silky lustre (Fig. 4).

Tyrosine is tasteless and odourless. When heated, it evolves an odour of burnt bones. Heated cautiously to 270° , it gives off carbon dioxide, and yields a white sublimate of hydroxyethylaniline, $C_2H_4(OH).NH.C_6H_5$.

Tyrosine is soluble in 2,450 parts of cold, or 154 parts of boiling, water. In hot ammonia and in acetic acid it dissolves unchanged,

and is deposited on cooling. It requires 13,500 parts of cold rectified spirit for solution, is not much more soluble on boiling, and is quite insoluble in absolute alcohol and in ether. Tyrosine may be separated from leucine by taking advantage of the fact of the solubility of the latter in glacial acetic acid mixed with an equal volume of alcohol. Tyrosine is decomposed by certain enzymes called tyrosinases, with the production of blackish colouring matters.

Natural tyrosine is laevorotatory. The value of $[\alpha]_D^{20}$ in 4% hydrochloric acid is -16° .

When fused with potassium hydroxide, tyrosine yields ammonia, acetic acid, and para-hydroxybenzoic acid, $C_6H_4(OH)COOH$.

When treated with strong nitric acid, tyrosine is converted into nitrotyrosine nitrate, from the solution of which ammonia precipitates free nitrotyrosine, $C_9H_{10}(NO_2)NO_3$, which crystallises from hot water in light yellow, very sparingly soluble needles, having a slightly bitter but not acid taste, and dissolving in alkali hydroxide solution, with deep red colour. *Dinitrotyrosine*, $C_9H_9(NO_2)_2NO_3$, is obtained by evaporating tyrosine with excess of nitric acid. It is a well-defined dibasic acid, forming golden-yellow plates, sparingly soluble in water, but readily in alcohol, having an acid but not bitter taste. The salts deflagrate on heating.

If Millon's reagent is added to a boiling aqueous solution of tyrosine, the liquid acquires a pink or rose-red colour, and red flakes are gradually precipitated. A method has been suggested for the quantitative estimation of tyrosine based upon the colour with this reagent.

When tyrosine is gently warmed with strong sulphuric acid, it dissolves, with transient red coloration, to form tyrosine-sulphonic acid, $C_9H_{10}(SO_3H)NO_3 + 2H_2O$. On diluting, boiling with barium carbonate or chalk, and gradually adding neutral ferric chloride to the neutral filtrate, a fine dark violet coloration is produced. This reaction, which is due to Piria, affords a very delicate test for tyrosine. Unfortunately, leucine somewhat interferes with it. Denigès' test for tyrosine is often very advantageous. The crystals to be tested are added to the reagent (consisting of formaldehyde 1, water 55, and sulphuric acid 55) and heated. A green colour develops, characteristic and permanent.

Tyrosine is not precipitated by phosphotungstic acid.

The hydrochloride of the ethyl ester forms long prisms which melt at 109° . The α -naphthyl-isocyanate forms small centrally grouped needles, m. p. 205° . The combination with β -naphthalene-sulpho-chloride, which does not respond to Millon's reaction, forms needles which melt to an oil at 120° . The amyl ester forms pale yellow needles melting at $68-70^{\circ}$; its hydrochloride at $181-182^{\circ}$. Tyrosine picrolonate blackens when heated to 260° , and is soluble in water (0.29:100; at $20-23^{\circ}$).

Tyrosine in the free condition or in peptide combination may be estimated colorimetrically as follows: (Folin and Denis, *J. Biol. Chem.*, **12**, 245; **14**, 457).

1 grm. of the dry protein is accurately weighed and transferred to a 500 c.c. Kjeldahl flask, 25 c.c. of 20% hydrochloric acid added, the flask closed by means of a Hopkins condenser made from a large test-tube, and the contents of the flask boiled for 12 hours over a micro-burner. At the end of this time the flame is removed, the contents of the flask transferred, on cooling, to a 100 c.c. flask and made up to volume. 1 or 2 c.c. of this solution are then transferred to a 100 c.c. flask, 5 c.c. of the tyrosine reagent¹ added, and, after 5 minutes, 25 c.c. of a saturated solution of sodium carbonate, and the mixture then made up to 100 c.c. with cold tap water. The maximum colour (blue) develops in about 10 minutes. Therefore the reading should not be made before this time has elapsed. Fading is very slow in the presence of the large excess of reagent used. As nearly at the same time as possible, a standard is prepared by treating 1 mg. of pure tyrosine with 5 c.c. of the phosphotungstic-phosphomolybdic reagent, then adding 25 c.c. of saturated sodium carbonate solution and making up to volume. The colours are compared by means of a Duboscq colorimeter, the standard solution being placed at 20 mm. As a standard solution, a solution of pure tyrosine in N/10 hydrochloric acid is used of such a concentration that 5 c.c. contain 1 mg. of tyrosine. In making the comparison of colour both solutions should, of course, be absolutely clear and contain no trace of precipitate; if any cloudiness is observed, the solution should be filtered before being used.

¹ A solution containing 10% of sodium tungstate, 2% of phosphomolybdic acid and 10% of phosphoric acid is made up by adding 100 grm. of sodium tungstate, 20 grm. of phosphomolybdic acid, and 50 c.c. of 85% phosphoric acid to 750 grm. of water, boiling for 2 hours under a reflux condenser, cooling, and diluting to 1,000 c.c.

Tryptophane and hydroxytryptophane also have been observed to give a slowly developing blue colour with this reagent (Abderhalden *J. Biol. Chem.*, **15**, 357; *Z. physiol. Chem.*, **83**, 468; **85**, 91).

The following reagents have also been proposed for the estimation of tyrosine and other amino-acids alone and in mixtures with other substances:—

*Phenol Reagent of Folin and Denis.*¹—This is prepared by boiling a solution of 15 grm. of molybdenum trioxide and 10 grm. of sodium hydroxide in 200 c.c. of water until there is no odour of ammonia. 100 grm. of sodium tungstate, 50 c.c. 85% phosphoric acid, 100 c.c. of conc. hydrochloric acid, and water are added to make a volume of 800 c.c., the whole boiled for 10 hours under a reflux condenser, a few drops of bromine added to decolorise it, and the liquid boiled without condenser to remove excess of bromine. The solution is then cooled, filtered and diluted to 1,000 c.c.

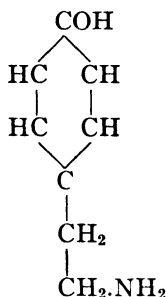
Uric Acid Reagent of Folin and Denis.—This reagent is prepared by boiling 100 grm. sodium tungstate with 80 c.c. of 85% phosphoric acid and 700 c.c. of water for not less than 2 hours, and diluting to 1 litre.

No coloured substances are formed by the action of the phenol reagent on arginine, lysine, aspartic acid, alanine, phenyl-alanine, histidine, glutamic acid, proline, leucine, valine, glycine, asparagine, cystine, isoleucine, and serine. Tyrosine and tryptophane give coloured substances.

Uric acid gives a colour with the phenol reagent about half as strong as does tyrosine. With a mixture of tyrosine and uric acid, therefore, the total colour is first determined, the uric acid then destroyed by hydrogen peroxide and sodium hydroxide, the phenol reagent added again, and the tyrosine determined alone (*Biochem. Z.*, 1918, **88**, 283).

The estimation of tyrosine and other amino-acids in mixtures will be given in connection with the description of these individual substances.

¹ *J. Biol. Chem.*, **51**, 421 (1922). The methods for the quantitative estimation of tyrosine and tryptophane have been still further improved. Cf. J. M. Looney (*J. Biol. Chem.*, 1926; **69**, 519; and O. Folin and V. Ciocalten, *J. Biol. Chem.*, 1927, **73**, 627).

Tyramine (*p*-Hydroxyphenylethylamine) $C_8H_{11}OH$; *i. e.*,

Tyramine has attracted considerable interest in recent years because of its marked physiological actions. It is formed in small amounts when ordinary putrefactive organisms are allowed to act upon protein in the absence of utilisable carbohydrates. It has been found in autolysed pancreas, etc., and also in various kinds of cheese. It is the chief pressor constituent in extracts of putrid meat; also in certain extracts of ergot. Injected intravenously into dogs, it causes a rapid and decided rise in blood pressure. It is probably identical with *mydine*, one of Brieger's ptomaines.

Tyramine may be separated from a mixture by fractionation with silver nitrate and barium hydroxide and separation as chloroplatinate from the lysine fraction. Or it may be extracted from its sodium carbonate solution with amyl alcohol.

Tyramine, if available in quantity, may be purified by distillation. It boils at $161-163^\circ$ under 2 mm.p. pressure, and at $175-181^\circ$ under 8 mm. pressure. It may be crystallised from boiling xylene and forms colourless hexagonal leaflets, m. p. 161° , soluble in 95 parts water at 15° and 10 parts boiling ethyl alcohol. The hydrochloride is very soluble in water, but crystallised from concentrated hydrochloric acid melts at 268° . The phosphate forms white prisms, m. p. $209-210^\circ$; the picrate, short prisms, m. p. 200° ; the chloroplatinate, six sided leaflets; the 3-nitro derivative, yellow to orange prisms, m. p. 217° with decomposition; the 3,5-dinitro derivative, orange red powder decomposing at 290° ; the 3-amino derivative, m. p. $145-147^\circ$; the 3-hydroxy hydrochloride compound, m. p. 207° with decomposition; and the N-monobenzoyl derivative, hexagonal plates, m. p. 162° . The dibenzoyl compound is the most character-

istic derivative and is used for purifying the compound. It is formed readily by the Schotten-Baumann reaction, and, recrystallised from alcohol, melts at 170° .

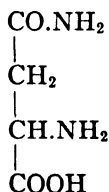
Tyramine is formed in a yield of 50% of the theoretical by heating tyrosine at 270° at 12–25 mm. pressure. It may also be obtained by reduction of *p*-hydroxy-phenyl-methyl-cyanide.

With phosphotungstic acid, large orange-red octahedra are formed by slow crystallisation.

Tyramine gives Millon's and Mörner's test for tyrosine, but gives no colour with triketohydrindene hydrate. It is transformed into *p*-hydroxyphenylacetic acid by perfusion through liver.

Tyrosine and tyramine may be estimated quantitatively by interaction in alkaline solution with *p*-phenyl-diazonium-sulphonate. The colour so obtained is intensified and stabilised by the addition of sodium hydroxide and hydroxylamine hydrochloride. An intense bluish-red colour is obtained. The method permits of the estimation of 0.001 mg. substance and is said to be accurate to 0.5–1.5%. In a mixture containing phenolic bodies, phenols and cresols are first removed by distillation with steam, the aromatic hydroxy acids are then extracted with ether from the acidified liquid, then tyramine is removed by extraction with amyl alcohol from the solution to which sodium carbonate had been added, and tyrosine is estimated in the liquid remaining (L. M. T. Hanke and K. K. Koessler, *J. Biol. Chem.*, 50, 235 (1922)). The phenols, apart from tyrosine and tyramine, may be estimated by means of the yellow to red colour they give with *p*-phenyl-diazonium sulphonate.

Asparagine.—Aminosuccinamic Acid.



Asparagine was discovered in 1805 in the juice of asparagus. It exists ready-formed in many other plants, including marsh-mallow,

comfrey, chestnuts, potatoes, the leaves of the deadly night-shade, liquorice-root, dahlia-tubers, and is present in comparatively large quantity in the roots of *Robinia pseudacacia*. Asparagine is also found in the milky juice of the lettuce, and in the young shoots of vetches, beans, peas, and other leguminous plants, though the seeds of these contain no trace of asparagine. The quantity of asparagine diminishes with the progress of the growth of the plant, and disappears entirely when the seeds are formed. Boussingault found asparagine to be constantly present in plants grown in the dark.

Asparagine may be prepared by dialysing the juice of asparagus, marsh-mallow, or *Scorzonera hispanica*, concentrating the dialysate to a syrup, and allowing it to stand for some days, when the asparagine will separate in crystals. From liquorice, asparagine may be prepared by exhausting the root with water, boiling to coagulate albumin, treating the filtrate with acetic acid to precipitate glycyrrhizic acid, and adding lead acetate to the filtered liquid, to throw down phosphates, malates, colouring matter, etc. The filtrate, evaporated to a small bulk, deposits crystals of asparagine after standing for some days. Asparagine may also be isolated by treating the filtrate from the lead precipitate with mercuric nitrate and decomposing the resulting compound with hydrogen sulphide. This plan is especially useful in the presence of soluble carbohydrates, which prevent the crystallisation of the asparagine.

Asparagine forms hard, transparent, rhombic prisms, which have a sp.gr. of 1.519. The crystals belong to the orthorhombic system, and exhibit left-handed hemihedry. They contain one molecule of water, which is lost at 100°. The crystals grate between the teeth, and have a slightly cooling, sickly taste.

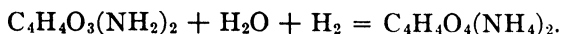
Asparagine is moderately soluble in cold water (1:82 at 10°; 1:47 at 20°), but more readily on boiling (1:1.9).¹ It dissolves freely in acid and alkaline liquids. It is insoluble in cold absolute alcohol, and almost insoluble on boiling, and is not dissolved by ether, or by fixed or volatile oils.

Asparagine is optically active, but the extent and direction of the rotation depend on the solvent. Thus a solution of asparagine in water has a specific rotation of about -6°; but by addition of alkalies the activity is increased, in ammonical solution the rotation

¹ The solubility of asparagine in cold water is given very variously due to impurities, the statements ranging from 1 in 12 to 1 in 300.

being -11° . In hydrochloric acid solution, (1 mol. HCl), on the other hand, asparagine exhibits a dextro-rotation of about $+26^{\circ}$. Addition of a small quantity of acetic acid to the aqueous solution of asparagine decreases the laevo-rotation, and on further addition of acid the liquid becomes dextrorotatory. (For data, see Landolt, *Optische Drehungsvermögen*.)

The aqueous solution of asparagine has a faintly alkaline reaction to litmus. When quite pure it can be kept without change, but in presence of protein matter and bacteria the solution soon ferments, the asparagine being completely converted into ammonium succinate:

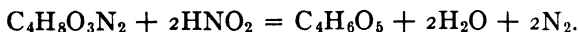


A similar change results when asparagine is taken internally, the urine, after asparagus has been eaten, acquiring a peculiar odour and containing ammonium succinate.

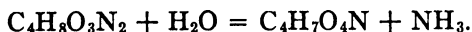
Asparagine exhibits both an acid and a basic function. The hydrochloride, $\text{C}_4\text{H}_3\text{O}_3\text{N}_2\cdot\text{HCl}$, forms large, readily soluble crystals. The salt $\text{Cu}(\text{C}_4\text{H}_3\text{O}_3\text{N}_2)_2$ is obtained by treating a solution of asparagine with cupric hydroxide or cupric acetate.

Asparagine reduces Fehling's solution on boiling, which reaction distinguishes it from glutamine.

When asparagine is dissolved in cold nitric acid (free from nitrous acid) it is converted into aspartic acid and ammonium nitrate; but if nitrous acid is present, or if nitric oxide or nitrous fumes are passed into the solution, the aspartic acid is converted into malic acid, with evolution of nitrogen, the reaction being, according to Sachsse and Kormann:



The most characteristic reaction of asparagine is its conversion into aspartic acid and ammonia by treatment with alkalis or mineral acids. The change readily occurs when asparagine is boiled with water and lime, baryta, or litharge; or with dilute hydrochloric or sulphuric acid:



The reaction might possibly be made quantitative, but B. Schulze has shown that there is a tendency to further decomposition if the

action is too prolonged. Boiled with water alone at the atmospheric pressure for 12 hours, only 2% of the asparagine was converted into ammonium aspartate. Under higher pressure the conversion was much greater. Milk of lime had no action in the cold after 24 hours, but on boiling with lime or barium hydroxide the action was much more rapid. When a large excess of baryta was used, the conversion was complete in 1 hour, but on continuing the treatment some hours more a further elimination of ammonia occurred, with formation of malic acid. Boiling with water containing one-tenth of its volume of strong hydrochloric acid effected complete conversion in one hour, action on the aspartic acid occurring if the treatment was further prolonged. Schulze also obtained good results by treating 2 grm. of asparagine with 5 c.c. (= 8.79 grm.) of pure sulphuric acid and 100 c.c. of water, and boiling under a reflux condenser for 2 hours. The cooled liquid was nearly neutralised with soda and distilled with magnesia. The ammonia found in the distillate was fairly in accordance with theory.

A method of determining asparagine in plant-products has been based on this reaction by R. Sachsse (*J. prakt. Chem.*, [ii], 6, 118); but for this purpose it is necessary previously to get rid of various co-occurring matters. Sachsse boils 10 grm. of the powdered substance for 15 minutes with 200 c.c. of a mixture of equal volumes of alcohol and water under a reflux condenser. 5 c.c. of a cold saturated solution of mercuric chloride¹ in alcohol are diluted with an equal measure of water and added to the decoction while still hot, and the liquid filtered, the residue being washed first with proof-spirit and then with cold water.² The filtrate is evaporated to dryness, the residue taken up in the minimum quantity of hot water (not more than 50 c.c.), and hydrogen sulphide passed through the filtered liquid, without filtering. The filtrate from the precipitated mercuric sulphide is then brought to a volume of 100 c.c., 10 c.c. of hydrochloric acid added, and the liquid boiled under a reflux condenser for 1 hour. The liquid is neutralised with soda and distilled with magnesia, the ammonia in the distillate being determined by titration with standard acid. 17 parts of ammonia or 14 of nitrogen, resulting from the treatment with hydrochloric acid, correspond to 150 parts of crystallised asparagine originally present.

¹ Schulze (*Ber.*, 15, 2255) employs mercuric nitrate in place of mercuric chloride for the precipitation of asparagine, and for its separation from carbohydrates.

² The washing may be avoided by making up the liquid to 500 c.c. passing it through a dry filter, and evaporating 400 c.c. of the filtrate (= 8 grm. of material).

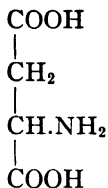
Estimations of asparagine by Sachsse's method in young lupines were found by Schulze and Barbieri to agree very nearly with the quantity obtained by crystallisation.

For the isolation of asparagine and glutamine from vegetable juices and extracts, E. Schulze (*Z. anal. Chem.*, **22**, 325) precipitates the liquid with basic lead acetate. The filtered solution is then treated with a neutral solution of mercuric nitrate (best made by adding sodium hydroxide to an acid solution until it no longer reddens methyl-orange). The white flocculent precipitate is filtered off, washed with cold water, and decomposed with hydrogen sulphide. The filtered liquid, boiled to free it from hydrogen sulphide, will, if asparagine or glutamine is present, evolve ammonia when boiled with alkali hydroxide and will dissolve cupric hydroxide to a deep blue solution. Allantoin is also precipitated by mercuric nitrate, but does not dissolve cupric hydroxide, and is precipitated on adding silver nitrate and ammonia. Xanthine, if present, will also be thrown down by the mercury. For the actual isolation of the amides, the filtrate from the mercuric sulphide precipitate should be neutralised with ammonia and evaporated to a small bulk, when asparagine and glutamine will be deposited in crystals on cooling. Or the original plant-juice, after boiling and filtering from coagulated proteins, may be acidified with sulphuric acid, and the peptones and ammonia precipitated with phosphotungstic acid. After standing 2 hours, the precipitate is filtered off, and the asparagine and glutamine estimated in the filtrate by boiling with dilute hydrochloric acid, and determining the ammonia formed by distillation with magnesia.

Other amino-compounds occur in plants, and are more or less liable to be estimated as asparagine unless special means are taken to separate them. E. Schulze, to whom the existing knowledge on the subject is largely due, finds the exact nature of the amino-compounds to vary with the plant under examination, and its age and conditions of life. In the *Caryophyllaceae* and *Filices* asparagine is entirely replaced by its homologue glutamine.¹

¹ In *Lupinus luteus*; Schulze found asparagine, phenylalanine, amino-valeric acid, arginine, choline, and xanthine-like substances; in *Cucurbita pepo*, glutamine, asparagine, leucine, tyrosine, arginine, choline, vernine, and xanthine-like substances; in *Vicia sativa*, asparagine, phenylalanine, leucine, aminovaleric acid, guanidine, choline, and betaine. This does not indicate that in plant-metabolism the protein molecule breaks down in different ways, it being contended that the disintegrative metabolism of protein is qualitatively the same, but varies quantitatively. This view is supported by experiments on plants of the same kind, but of different ages. Schulze suggests that in some plants certain varieties of nitrogenous crystalline compounds are used more in nourishing the tissues, while in other plants other compounds are more advantageous, and so are used up first.

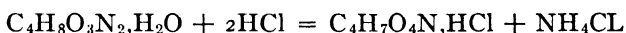
Aspartic acid,



has the constitution of an aminosuccinic acid.

It occurs in beetroot molasses, doubtless as a product of the decomposition of asparagine, in spent wine lees or vinasse, and in other vegetable juices.¹ It is also formed by boiling albumin or casein with dilute sulphuric acid, by the action of stannous chloride on horn, by treating proteins with bromine, etc. It is secreted pre-formed by certain sea-snails.

Aspartic acid is best prepared by the hydrolysis of asparagine. H. Schiff (*Ber.*, 17, 2929) recommends that 100 grm. of asparagine should be boiled for 2 or 3 hours under a reflux condenser with 408 c.c. of hydrochloric acid, containing 48.65 grm. of hydrogen chloride; that is, sufficient for the reaction:



To the cooled solution are added about 200 c.c. of ammonia solution, containing an amount of ammonia sufficient to neutralise just one-half the acid previously employed. (The other half has been neutralised by the ammonia formed in the hydrolysis.) On cooling the liquid and allowing it to stand, aspartic acid separates in colourless crystals.

Aspartic acid forms small rectangular plates, having a sp. gr. of 1.66. It dissolves in about 250 parts of cold water, or in 19 of boiling water, and hence is much less soluble than asparagine. In alcohol it is nearly insoluble.

The free acid and its salts with alkaline metals are laevorotatory; free acid $[\alpha]_D^{20} = -3.63^\circ$; Li salt -4.86° ; NH_4 salt -7.60° ; Na salt -9.09° ; K salt -14.20° . In 4% HCl solution, it is dextrorotatory, $[\alpha]_D^{20} = +25.7^\circ$.

¹ For the isolation of aspartic acid, the boiling liquid containing it should be treated with carbonate of barium or lead, and alcohol added as long as further precipitation occurs. The precipitate is treated with water, and the barium or lead aspartate reprecipitated by addition of alcohol. The precipitate is again dissolved in water, the barium or lead precipitated with dilute sulphuric acid, and the filtered liquid evaporated to the crystallising point. The crystals are purified by treatment with 60% spirit, and the residue boiled with water, when pure aspartic acid crystallises out on cooling.

The inactive aspartic acid was synthesised by heating fumaric acid with ammonia, as well as by other methods, while by the action of ammonia on *l*-bromsuccinic acid the *d*-acid was obtained.

Aspartic acid forms a series of crystallisable salts with bases. The cupric salt, $\text{CuC}_4\text{H}_5\text{NO}_4 \cdot 4\frac{1}{2}\text{H}_2\text{O}$, forms blue needles, soluble in hot water, but very sparingly soluble in cold water, (1:2800).



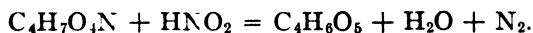
FIG. 5.—Aspartic acid. $\times 80$. (From Keenan, *J. Biol. Chem.*, 1924, **62**, 163.)

This fact may be employed for the detection and isolation of aspartic acid, solutions of which may be precipitated by cupric acetate (see Lewinsky, *Chem. Centralb.*, 1894, **1**, 53).

With phosphotungstic acid, stubby microscopic crystals may be obtained.

Aspartic acid reduces Fehling's solution.

Aspartic acid is not decomposed by alkaline hypobromite solution, but by treatment with nitrous acid it is converted into malic acid, with evolution of nitrogen:

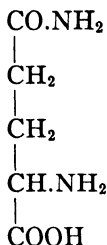


This reaction was employed by Sachsse and Kormann for the estimation of aspartic acid, and, indirectly, of asparagine. In practice, sodium nitrite and dilute sulphuric acid are substituted for nitrous acid.

F. Meunier (*Ann. Agronomiques*, **6**, 275; *J. Chem. Soc.*, **40**, 761) finds that the estimation of asparagine by measurement of the nitrogen evolved by the action of nitrous acid is inaccurate. He has devised the following process, which depends upon the production of potassium aspartate and ammonia when asparagine is treated with potassium hydroxide. The crushed, dried, and weighed substance is placed in a little bag with meshes small enough to retain the starch. This is placed in a porcelain dish, exhausted with boiling water, the filtered solution is heated with subacetate of lead to precipitate proteins and leucine, and the excess of lead is removed from the filtrate by means of sodium hydrogen carbonate. The filtrate from the lead carbonate is distilled with alkali hydroxide, and the ammonia in the distillate titrated with standard acid. Since ammonium salts are present, they must be separately estimated and subtracted.

The crude crystals, collected as previously described, are to be purified by passing through the cupric salt. The benzoyl compound crystallises in shining plates which lose their water of crystallisation at 110° , and melt at $184-185^{\circ}$.

Glutamine. Aminoglutamic Acid



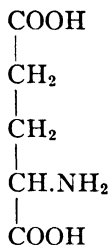
Glutamine is the higher homologue of asparagine, and co-exists with it in beetroot, pumpkins, and the shoots of vetch. In the families *Caryophyllaceae* and *Filices* glutamine completely replaces asparagine. Glutamine is also a product of the action of dilute acids or barium hydroxide on proteins.

Glutamine forms slender anhydrous needles, soluble in 25 parts of cold water and much more readily at the b. p. It is insoluble in absolute alcohol. The aqueous solution (4 grm. per 100 c.c.) is optically inactive, but the solutions in hydrochloric and oxalic acids are dextrorotatory.

When heated with alkalis or dilute mineral acids, glutamine yields ammonia and glutamic acid.

Glutamine does not reduce Fehling's solution, but dissolves cupric hydroxide to a deep blue solution, a crystallisable compound being formed analogous to that yielded by glycocholl (page 225). Glutamine forms an insoluble compound with mercuric nitrate, a fact utilised by Schulze and Bosshard to isolate it from the juice of beetroot (*Ber.*, 16, 312; 18, 290) (See page 268).

Glutamic Acid, α -Amino-glutaric acid,



is the higher homologue of aspartic acid, bearing the same relation to normal glutaric acid that aspartic acid bears to succinic acid. Glutamic acid has been isolated from molasses after the sugar has been removed by the strontium process, and is formed, together with aspartic acid, by boiling proteins with dilute sulphuric acid.

Glutamic acid forms orthorhombic tetrahedra or octahedra, m. p. 202°. It dissolves in about 100 parts of cold water, and is much less soluble in alcohol. The solutions are acid, and have an astringent taste, with a peculiar after-taste. Glutamic acid differs from aspartic acid in yielding no precipitate with lead acetate even after the addition of ammonia; but the lead salt may be precipitated by adding alcohol to the concentrated filtrate from any precipitate produced by basic lead acetate. Glutamic acid is also distinguished from aspartic acid by not reducing Fehling's solution on heating. It forms a characteristic copper salt which is

very sparingly soluble in cold water. This fact may be used for the isolation of glutamic acid.

Glutamic acid rotates the plane of polarised light to the right, in the case of the naturally occurring acid obtained through the action of acid; in water $[\alpha]_D^{20} = +12^\circ$, but much increased by the presence of acid; thus in 10% hydrochloric acid $[\alpha]_D^{20} = +31^\circ$.

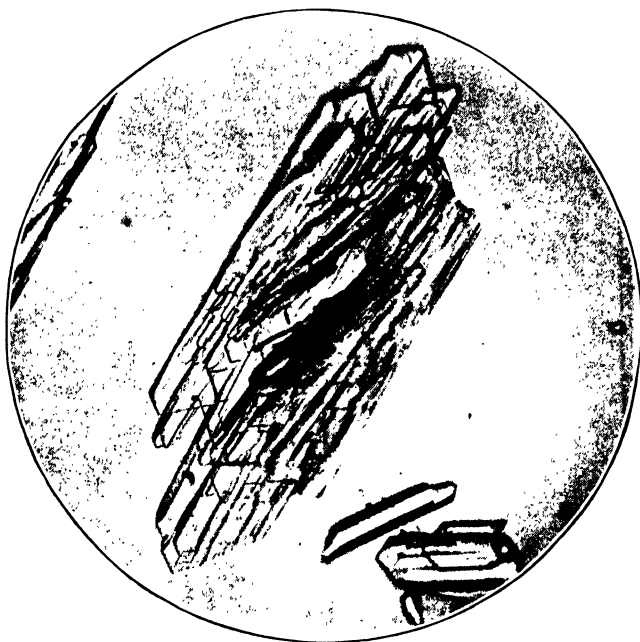


FIG. 6.—Glutamic acid. $\times 100$. (From Keenan, *J. Biol. Chem.*, 1924, **62**, 163.)

The crude glutamic acid, obtained as previously described, is purified by recrystallisation from hot hydrochloric acid. The α -naphthyl-iso-cyanate forms long thread-like needles, m. p. 236° . Benzoyl-*d*-glutamic acid forms plates, m. p. 139° . The picrolonate of *dl*-glutamic acid melts at 194° and dissolves in water, 2.37 parts to 100.

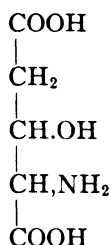
When glutamic acid is heated to 180 – 190° , or boiled in aqueous solution, pyrrolidone-carboxylic acid is formed. This, when heated with strong hydrochloric acid, reverts to glutamic acid. It is

possible that this reaction occurs to some extent in the hydrolysis of proteins.

Glutamic acid has been synthesized by the reduction of α -is-nitroso-glutaric acid.

The separation of glutamic and aspartic acids from the products of hydrolysis of proteins by hydrochloric acid may be effected, after evaporation under diminished pressure to remove the hydrochloric acid and to concentrate the solution, by precipitation of their calcium salts by addition of calcium oxide and alcohol (F. W. Foreman, *Biochem. J.*, 1914, **8**, 463). It was also found that glutamic and aspartic acids are practically insoluble in glacial acetic acid.

β -Hydroxyglutamic acid, α -Amino- β -hydroxyglutaric acid,



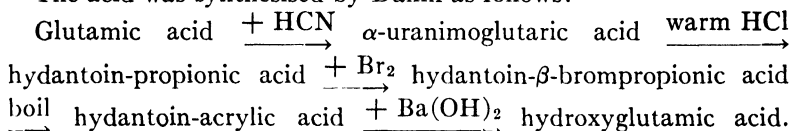
was discovered by Dakin among the hydrolysis products of casein, by the butyl alcohol extraction procedure. It was found in 10.5% yield. It was later found in smaller proportions in a number of additional proteins. The acid, as well as its salts, is extremely soluble in water, and in the separation after removal of the other dibasic amino-acids by calcium and lead precipitation, can be precipitated as the silver salt in dilute alkali. The free acid separates from its concentrated solution (clear yellowish syrup) as thick prisms. It is soluble in its own weight of water, slightly soluble in methyl alcohol, and practically insoluble in ethyl alcohol, ether, and ethyl acetate. A mixture of solvents may therefore be used in its purification by recrystallisation. It melts at $140-150^\circ$, with partial conversion into hydroxy-pyrrolidone carboxylic acid.

The aqueous solution shows a small dextrorotatory power, increased on the addition of hydrochloric acid.

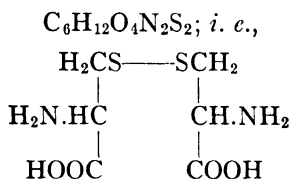
A number of salts and derivatives of the β -hydroxyglutamic were prepared and colour reactions described. The most significant of these is a clear fluorescent green colour obtained with α -naphthol

in concentrated sulphuric acid, and a clear reddish-purple colour turning brown on warming with resorcinol under the same conditions, all traces of nitrates being absent in both tests.

The acid was synthesised by Dakin as follows:—



Cystine. Dithio-diamino-dilactic acid.



Cystine is the leading constituent of rarely-occurring urinary and renal calculi. It is also met with as a sediment from urine. It may be prepared from such sediment, or preferably from the calculus when obtainable, by treating the substance with ammonia, and allowing the filtered liquid to evaporate spontaneously, when the cystine is deposited in characteristic colourless or pale yellow hexagonal tablets of sharp contour (Fig. 7), which are often superposed. Cystine is present in proteins, with a few exceptions, such as protamine and fibroin, which lack it.

Cystine is colourless, odourless, and tasteless. When heated, it ignites without melting, and burns with a greenish-blue flame, emitting a characteristic penetrating odour resembling that of hydrocyanic acid. Heated in a closed tube, cystine decomposes at 258° – 261° , giving off ammonia and yielding a distillate of disagreeable odour, and leaving a residue of carbon.

Cystine is quite insoluble in water, alcohol, and ether. It is readily soluble in ammonia (distinction from uric acid), in fixed alkali hydroxide and alkali carbonates, but not in ammonium carbonate. It is precipitated from these solutions by acetic acid. Maximum precipitation of the cystine takes place when the solution is at pH 3–6. Precipitation at pH 3 frees it from tyrosine. Cystine consequently dissolves in mineral acids and in oxalic acid, but not

in tartaric or acetic acid. The solution in hydrochloric acid containing 11.2% of HCl has a specific rotation of $[\alpha]_D^{20} = -224^\circ$.

Cystine forms unstable salts with acids, and is precipitated from solutions in acids by ammonium carbonate. The hydrochloride unites with mercuric chloride to form a crystalline compound which is nearly insoluble in water.

If a cold solution of cystine in ammonia is treated with ammonio-nitrate of silver, and the liquid then cautiously neutralised with

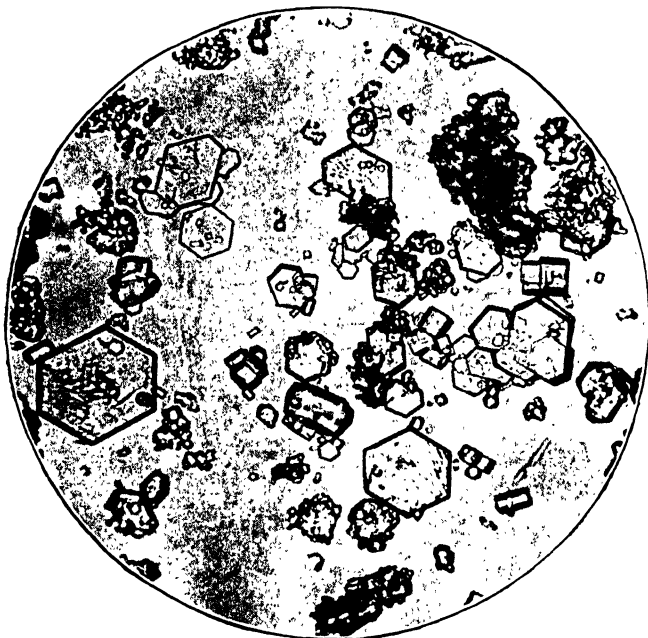


FIG. 7.—Cystine. $\times 100$. (From Keenan, *J. Biol. Chem.*, 1924, 62, 163.)

nitric acid, a canary-yellow precipitate is thrown down, but if the solution is heated silver sulphide is precipitated.

Its phosphotungstate forms pale sulphur-yellow small rhomboidal crystals.

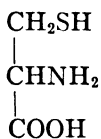
The phenyl isocyanate compound, m. p. 160° , boiled with 25% hydrochloric acid, forms the hydantoin derivative (by loss of water), m. p. $117-119^\circ$.

A method has been described by Plimmer, (*Biochem. J.*, 1913, 7, 311), for separating cystine and tyrosine from each other. The

mixture is warmed with absolute alcohol saturated with hydrochloric acid, and an equal volume of alcohol then added. Cystine is insoluble and may be filtered off, washed, dried, and weighed. Tyrosine readily forms the ester under these conditions and goes into solution. It may be recovered by diluting with 2 volumes of water, boiling for 8 hours, and neutralising with ammonia.

Folin and Denis have developed an accurate method for estimating cystine (*J. Biol. Chem.*, 1922, **51**, 421). Applied to proteins, it is as follows:—1 to 5 gm. of the dry protein are heated with 25 c.c. of 20% sulphuric acid under a Hopkins condenser for 12 hours, cooled and diluted to 100 c.c. From 1 to 10 c.c. are measured into a 100 c.c. volumetric flask, and 20 c.c. of saturated sodium carbonate solution and 10 c.c. of 20% sodium sulphate added. After 5 minutes 3 c.c. of the Folin and Denis uric acid reagent (see above) are added, the mixture allowed to stand for 10 minutes, diluted to the mark, and compared in a colorimeter with a standard prepared from 1 or 3 mg. cystine treated similarly.

When treated with granulated tin in hydrochloric acid solution, cystine is reduced to aminothiolic acid or cystein, $C_3H_7O_2NS$:



Cystein is a crystalline powder, soluble in water, in ammonia, and in acids. The aqueous solution is oxidised to cystine on exposure to the air.

By reaction with nitrous acid cystein yields pyruvic acid, $CH_3CO.COOH$.

When heated with nitric acid, cystine is decomposed, with production of a brown coloration.

When boiled with alkali hydroxides, cystine evolves ammonia. The solution then contains a sulphide, and hence gives a black precipitate on addition of lead acetate. The sulphur is not wholly converted into sulphide, even after many hours' boiling with alkali hydroxide.

For the detection of cystine in a calculus, the powdered substance should be dissolved in alkali hydroxide and acetic acid added to the hot solution, when cystine, if present, will separate on cooling,

and can be recognised by its crystalline form. Or the calculus may be treated with hot ammonia, and the filtered liquid evaporated to the crystallising point. Any xanthine will be dissolved out and deposited with the cystine. Goldmann and Baumann (*Z. physiol. Chem.*, 1888, 254) have proved that cystine or an allied substance is always present in urine. This method is based on the fact that when a few drops of benzoyl chloride are added to a solution of cystine in sodium hydroxide, a voluminous precipitate of shining plates of the sodium salt of benzoylcystine, $C_6H_{10}O_4N_2S_2Bz_2$, is formed. This compound is soluble in hot water, less soluble in cold, and quite insoluble when excess of sodium hydroxide is present. On adding a strong acid to the dilute solution, the liquid sets to a transparent jelly, but, on warming and standing, free benzoylcystine separates in dense flocks which can be separated by filtration. Benzoyl-cystine is a strong acid, almost insoluble in water, and but slightly soluble in pure ether, but more readily soluble in ether containing alcohol. In alcohol it dissolves, and crystallises from the solution in slender needles which tend to aggregate in cauliflower-like masses.

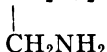
For the isolation of cystine from urine, Goldmann and Baumann recommend that 200 c.c. of the sample should be treated with 10 c.c. of benzoyl chloride and 70 c.c. of sodium hydroxide solution of 1.12 sp. gr., and the mixture shaken until the benzoyl chloride has dissolved. The precipitate (which consists of benzoyl compounds of urinary carbohydrates, mixed with phosphates) is filtered off, and the filtrate rendered strongly acid with sulphuric acid, and shaken with ether containing alcohol. The ethereal layer is separated, evaporated, and the residue boiled for 1 hour with sodium hydroxide and lead acetate. The lead sulphide produced is equivalent to about two-thirds of the cystine isolated, the cystine represented being three-fourths of the actual weight of lead sulphide obtained. From 200 c.c. of normal urine Goldmann and Baumann obtained 0.0025 gm. of lead sulphide, representing 0.0009 gm. of cystine for 100 c.c. of urine. In idiopathic cystinuria large amounts of cystine are found in the urine, independent of any formation of calculi, as the result of metabolic anomaly.

The crude cystine recovered in the method previously described should be purified, after removal of the tyrosine, by recrystallisation from hot alkaline solution and acidification with acetic acid. If it

will not crystallise, it may be purified by precipitation with benzoyl chloride. The benzoylcystine melts at 180–181°.

A distinctive test for cystein may be given in this connection (M. X. Sullivan). Cystein gives a red colour with sodium 1,2-naphthoquinone-4-sulphonate in the presence of alkali and a reducing agent such as sodium sulphite or $\text{Na}_2\text{S}_2\text{O}_4$ and sodium cyanide. Cystine gives the reaction slowly because of the gradual reduction of the cystine by the sulphite (more rapidly if NaCN is added to the cystine before the other reagents). The colour is not given by compounds containing either the SH or NH_2 group alone, by compounds containing NH_2 and S as in cystine, by mixtures of amino acids and compounds containing the SH group, by compounds containing both the SH and NH_2 groups, but far apart in the molecule as in reduced glutathione.

Taurine. Aminoethane-sulphonic acid.



Minute quantities of taurine are stated to exist in the juices of the lungs and of muscles, but its principal mode of occurrence is in the form of taurocholic acid, $\text{C}_{26}\text{H}_{45}\text{O}_7\text{NS}$, which is a characteristic constituent of the bile.¹ Taurine is formed in the animal body only in the liver, and its amount varies widely in the bile of different animals.

Taurine is best prepared by boiling ox-bile for some hours with dilute hydrochloric acid, separating the liquid from the resinous product, and precipitating the remaining traces of bile-acids with lead acetate. The filtrate is freed from lead by means of hydrogen sulphide, concentrated, and the taurine, which separates on cooling, purified by recrystallisation from water.

Taurine has been obtained synthetically by the following series of changes: Ethylene, C_2H_4 , is absorbed by fuming sulphuric acid, the product dissolved in water, neutralised with ammonia, and the solution evaporated to the crystallising point. The resulting

¹ Free taurine was found by Gorup-Besanez in the liver of a person who died from *arachnitis*. It has been detected in the liver in cases of jaundice, and has also been met with in the kidneys, lungs, and muscles. It is likewise present in the intestinal canal and in excrement, doubtless as a product of the decomposition of taurocholic acid.

ammonium isethionate, $\text{C}_2\text{H}_4\text{O}.\text{SO}_3(\text{NH}_4)$, when heated to 220° , yields taurine and water. Taurine has also been obtained by converting ethylene into glycol-chlorhydrin, and treating that substance in the following manner:

Glycol-chlorhydrin, $\text{HO}.\text{C}_2\text{H}_4.\text{Cl}$, heated with K_2SO_3 , gives potassium isethionate, $\text{HO}.\text{C}_2\text{H}_4.\text{SO}_2.\text{OK}$; which, distilled with PCl_5 , yields isethionic chloride, $\text{Cl}.\text{C}_2\text{H}_4.\text{SO}_2.\text{Cl}$, which, on heating with water, yields chlorethyl-sulphonic acid, $\text{Cl}.\text{C}_2\text{H}_4.\text{SO}_2.\text{OH}$; this, with ammonia at 100° under pressure, gives taurine, $\text{NH}_2.\text{C}_2\text{H}_4.\text{SO}_2.\text{OH}$.

Taurine crystallises in hard, six-sided prisms (Fig. 8), which crackle between the teeth. It melts at 240° , with intumescence

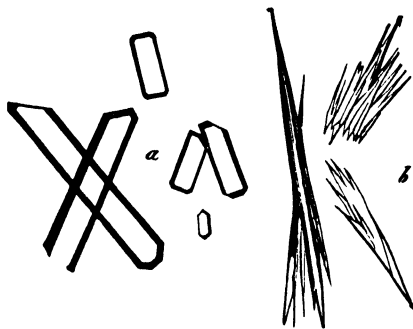


FIG. 8.—Taurine, *a*, well-formed six-sided prisms; *b*, irregular sheaf-like masses from an impure solution.

and evolution of sulphur dioxide, etc., leaving a difficultly combustible carbonaceous residue.

Taurine has a fresh taste, dissolves in 15 parts of water at ordinary temperatures, and is much more soluble at higher temperatures, but is only sparingly soluble in 5% alcohol (1:500), and is practically insoluble in absolute alcohol or ether.

Taurine has no acid reaction, but it forms soluble crystallisable salts with bases. It dissolves in hot dilute acids, and separates unchanged on cooling. When the solution of taurine is evaporated with alkali hydroxide, the whole of the nitrogen is evolved as ammonia, and the residue contains a sulphite and acetate of alkali metal. Fused with potassium hydroxide, the same products are obtained. If heated strongly with sodium carbonate, avoiding

access of air, taurine yields a product containing much sodium sulphide. Hence the solution of the mass in water blackens a silver coin, and evolves hydrogen sulphide when treated with an acid.

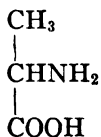
By reaction with nitrous acid, taurine yields isethionic acid.

Solutions of taurine are not precipitated by metallic salts ordinarily, nor by tannin. However, an insoluble white compound is formed on boiling taurine with freshly precipitated mercuric oxide.

For the demonstration or isolation of taurine in bile or tissue, the material should be boiled with 10% hydrochloric acid for 6 hours, and the hot solution filtered through animal charcoal. It is then concentrated to a small volume and again filtered hot to remove salts. The filtrate is next evaporated to dryness, the residue dissolved in 5% hydrochloric acid, and the taurine precipitated by the addition of 10 volumes of alcohol. The crystals should be purified by recrystallisation from hot water to which hot alcohol has been added. Taurine combines with mercuric oxide to form a white compound insoluble in the hot mixture. The sulphur content is the basis of the quantitative estimation.

The β -naphthalene-sulpho compound, m. p. 247° , may be used to separate and characterise taurine in urine and also in complex mixtures (*Z. physiol. Chem.*, 1916, **97**, 260).

Alanine. α -Amino-propionic acid.



Alanine occurs in the dextrorotatory form in nearly all animal and vegetable proteins, the albuminoids being especially rich in it. It has a sweetish taste, is very soluble in water, quite insoluble in alcohol. The specific rotation is $[\alpha]_D^{20} + 2.70$ in aqueous solution, but $+10^{\circ}$ in dilute hydrochloric acid. It dissolves cupric hydroxide on boiling, with the production of a soluble crystalline copper salt. It occurs, mixed with leucine and valine, in the distillate by the

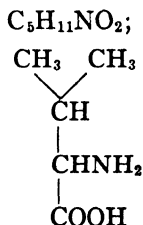
ester method, and must be separated from these by fractional crystallisation. Characteristic derivatives are the β -naphthalene-sulpho-*d*-alanine (fine clustered needles, m. p. 81°), the phenyl-iso-



FIG. 9.—Alanine. $\times 100$. (From Keenan, *J. Biol. Chem.*, 1924, **62**, 163.)

cyanate (needles melting at 168°), the naphthyl-iso-cyanate (small fine needles, m. p., 191°), and the picrolonate, m. p. 214° .

Valine. α -Amino-valeric acid.



Valine occurs in traces in most proteins, even in the protamines. It is soluble in water, and readily soluble in alcohol, especially methyl

alcohol. It has a sweetish taste, with a bitter after-taste. It dissolves cupric hydroxide on boiling; the copper salt is easily soluble in water, shows little tendency to crystallisation, and is soluble in methyl alcohol. The naturally occurring valine has $[\alpha]_D^{20} = +6.42^\circ$ in water and $[\alpha]_D^{20} = +28.8^\circ$ in 20% hydrochloric acid. It is obtained mixed with the crystals of leucine, from which it must be separated by fractional crystallisation. It forms thin,



FIG. 10.—Valine. $\times 100$. (From Keenan, *J. Biol. Chem.*, 1924, **62**, 163.)

narrow, rod-shaped plates characterised by jagged ends and long longitudinal slits. It forms no really well-characterised derivative; the hydantoin of the phenyl-iso-cyanate melts at 131° . The picrolonate melts at 180° and is soluble (1.2:100) in water.

A method for separating *d*-alanine (and glycine, if present) and *d*-valine, which are obtained in one of the fractions of the amino-acids obtained by the ester method of separation of the hydrolysis products from proteins, was devised by Levene and van Slyke (*J. Biol. Chem.*, 1913, **16**, 103). The method is based on the fact that a crystalline salt is formed by the *d*-alanine with phospho-

tungstic acid in a ratio of 1 to 14 by weight, and with solubility 0.15 grm. alanine per 100 c.c., in a solution containing 20 grm. phosphotungstic acid in 100 c.c. 10% sulphuric acid, whilst under the same conditions the solubility of *d*-valine is 1.21 grm. per 100 c.c. By alternate crystallisation of valine as the free amino-acid (with the addition of acetone to a concentration of 50–60%), and of alanine as the phosphotungstate, subsequently removing this phosphotungstic acid with lead acetate, a practically quantitative separation of a mixture of the two amino-acids can be effected.

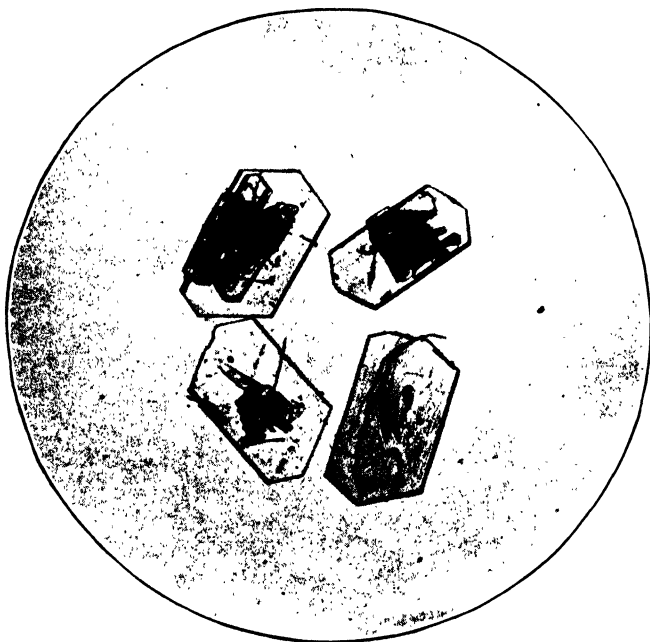
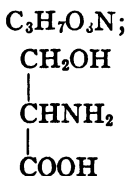


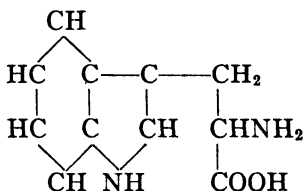
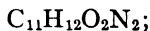
FIG. 11.—Serine. $\times 10$. (From Keenan, *J. Biol. Chem.*, 1924, **62**, 163.)

Serine. α -Amino- β -hydroxypropionic acid.



Serine exists widespread in animal and vegetable proteins, in amounts larger than usually reported, on account of the difficulties in the quantitative isolation. The naturally occurring form is usually racemic. It has been prepared synthetically by Fischer and Leuchs by the reaction between ammonia, hydrocyanic acid and glycollic aldehyde, and the *d*- and *l*-serine may be obtained from the racemic amino-acid. Serine is obviously derived in nature from alanine, and on reduction yields alanine. It is the amine of glyceric acid. Serine is rather soluble in water, quite soluble in alcohol. It has a peculiar sweetish taste, with an unpleasant after-taste. In the ester method it is obtained as the final amino-acid in the fraction distilling over at the highest temperature. It is fairly soluble in water, 1 to 23 at 20°, and crystallises in platelets or prisms. The β -naphthalene-sulpho-serine tends to be amorphous, but on repeated crystallisation from hot alcohol forms small needles which melt at 214°. The phenyl-iso-cyanate-serine forms fine, clustered needles, soluble in both water and alcohol, melting at 168°. The picrolonate decomposes at 265°, and dissolves in water, 0.98 parts in 100.

Tryptophane. Indole- α -amino-propionic acid.



Tryptophane exists, in small quantities, in nearly all proteins, the keratins and collagens and the protamines being devoid of it. It is an amino-acid of the greatest importance in metabolism, and no protein devoid of it can be a complete protein in tissue-building. It is the mother substance of the indole, which as indole and skatole and indican and skatoxyl appear in the fæces and urine. The processes of digestion simply split off the tryptophane; it is bacterial processes that split this molecule and set the indole free. It has been synthesised by Ellinger and Flammand (*Ber.* 1907, 40, 3029).

The β -indole-aldehyde is first condensed with hippuric acid to form lactimide, which, on being boiled with dilute alkali hydroxide, takes up water and yields indoxyl- α -benzoyl-amino-acrylic acid. The benzoyl group is then split off by the action of sodium ethoxide, following which reduction yields tryptophane. The naturally occurring tryptophane is the *l*-form, $[\alpha]_D^{20} = -30^\circ$. In hydrochloric



FIG. 12.—Tryptophane. $\times 100$. (From Keenan, *J. Biol. Chem.*, 1924, **62**, 163.)

acid and in potassium hydroxide, it is dextrorotatory, and the racemic compound is easily formed. Tryptophane has a bitter taste; it is quite soluble in even cold water, almost insoluble in alcohol. It melts at about 255° , when slowly heated.

The chemical processes necessary for the isolation of tryptophane are somewhat complicated. If it is to be sought for in a protein, the protein must be digested with an enzyme, not hydrolysed with acid. The protein is to be suspended in water, trypsin added, the solution made alkaline, toluene added, and the mass allowed to stand for a

week or more. From time to time the bromine test may be applied, and from this one may judge when the yield is complete. Finally the mass is heated to 80° , filtered, sulphuric acid added to make a concentration of 5%, and a 10% solution of mercuric sulphate in 5% sulphuric acid added in excess. The heavy precipitate is collected on a filter, washed with 5% sulphuric acid with the aid of suction, suspended in water, heated, and the mercury precipitated with hydrogen sulphide. The mixture is filtered, and the hydrogen sulphide expelled with carbon dioxide. Sulphuric acid is again added to make the concentration 5%, and a solution of mercuric sulphate carefully added until a clinging precipitate is formed which does not seem to increase. This consists of cystine and other impurities, and should be removed by filtration. To the filtrate is then added excess of the mercuric sulphate, the precipitate collected upon a Buchner funnel, and washed with 5% sulphuric acid until the wash water gives no reaction with Millon's reagent. The precipitate is then suspended in water, the mercury precipitated from the hot solution with hydrogen sulphide, filtered, the sulphuric acid removed with barium hydroxide, and the final filtrate concentrated at 40° at diminished pressure. A little alcohol is then added, and the concentration continued under diminished pressure at 40° , until signs of crystallisation appear. After standing on ice for several days, the crystals are suspended in warm water and recrystallised from 50% alcohol. One recrystallisation may be necessary. If pre-formed tryptophane is to be sought for in a material, one would begin where the first precipitation with mercuric sulphate is effected. It crystallises in soft, 6-sided crystals.

The extraction and estimation of tryptophane by the Dakin butyl alcohol method is considerably simpler and more reliable than the older methods. It may be carried out as follows (with caseinogen as example):—187 grm. of caseinogen were digested for 7 days with active pancreas extract in 2 litres of a 1% sodium carbonate solution. The mixture was then precipitated once with mercuric sulphate solution, the precipitate decomposed with hydrogen sulphide, sulphuric acid exactly removed with barium hydroxide, and the solution concentrated under diminished pressure to about 30 c.c. It was then extracted with butyl alcohol in a small extractor. A separation of tryptophane on the sides of the extraction flask was soon noted, and at the end of 2 hours' extraction, 1.5 grm.

had separated out. The extraction was practically complete after 12 hours. 3.15 grm. of tryptophane crystallised from the butyl alcohol extract, and, after washing with ether, was remarkably pure, considering the simple treatment. A simple crystallisation from alcohol gave a very pure product.

When a solution of tryptophane is mixed with bromine water a rose-red colour develops. A pine stick moistened with hydrochloric acid and then washed with water is immersed in a solution of tryptophane; on drying, the purple colour of the pyrrole reaction develops. If an aqueous solution of glyoxylic acid is mixed with a solution of tryptophane, and strong sulphuric acid then poured upon the mixed solution, a red-violet colour will appear at the contact of the two layers (Hopkins and Cole, *Proc. Roy. Soc.*, 68). When heated, indole and skatole are formed; many bacteria also form indole from a culture medium containing tryptophane. β -Naphthalene-sulpho-tryptophane forms fine needles, m.p. 180° ; the α -naphthyl-iso-cyanate-tryptophane melts at 158° ; the benzene-sulpho-tryptophane forms small leaves which melt at 185° . A few drops of a 5% solution of vanillin in 95% alcohol, when added to a solution of tryptophane in the presence of sufficient sulphuric acid, produce a deep red to purple coloration.'

In view of the importance of tryptophane for changes occurring in biological processes, several new methods of estimation and separation will be given. A quantitative method has been proposed (Herzfeld, *Biochem. Z.* 1913, 56, 258) based upon the blue colour developed slowly (30 hours) by tryptophane with a solution containing 20 grm. of *p*-dimethylaminobenzaldehyde, 500 c.c. concentrated hydrochloric acid, and 500 c.c. water. For colour comparison, an ammonical copper sulphate solution was suggested. It is said that 0.1 mg. of tryptophane may be determined in 100 c.c. of solution in this way.

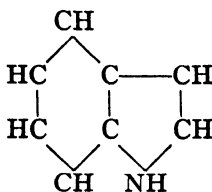
A reaction which is adaptable for quantitative work may be carried out as follows (E. Komm and E. Böhringer, *Z. physiol. Chem.*, 1923, 124, 287):—Five c.c. of tryptophane or protein solution are treated with 5 c.c. of 15% HCl containing 6 c.c. of 0.1% formaldehyde per 500 c.c., 10 c.c. of concentrated sulphuric acid are added, and the mixture shaken carefully until no bubbles of HCl appear. A blue-violet colour develops in the presence of tryptophane. One part of tryptophane in 75,000 gives a satisfactory

reaction. To employ the test in a quantitative manner, the protein is weighed in a colorimeter cup, treated as described, and the colour compared with that given by a known amount of tryptophane. In general, the accuracy is within 2-4% of the theoretical value. Hydrogen peroxide and hydrogen interfere with the reaction. The colour does not fade within 24 hours.

The recent procedure of Folin and Denis (*J. Biol. Chem.*, 1922, **51**, 421) for the estimation of tyrosine and tryptophane in proteins should also be given. One grm. of the dried protein is heated with 3.5 grm. of $\text{Ba}(\text{OH})_2 \cdot 8\text{H}_2\text{O}$ in 25 c.c. water for 40-48 hours under a Hopkins condenser. 30 c.c. of 20% sulphuric acid are then added, the mixture heated in boiling water for 30-60 minutes, cooled, diluted to 100 c.c., and filtered. From 1 to 8 c.c. are placed in a graduated centrifuge tube, 2 c.c. of 10% HgSO_4 in 5% H_2SO_4 and enough additional 5% H_2SO_4 to bring the volume to 10 c.c. are added. After shaking, standing for 2 hours and centrifuging, the supernatant liquid is poured off and used for the tyrosine estimation. Enough 5% H_2SO_4 is added to the residue to make a volume of 10 c.c., the mixture shaken and centrifuged again. The liquid is decanted, 10 c.c. water and 4 c.c. 5% NaCN solution added, rinsed into a 100 c.c. volumetric flask, diluted to about 50 c.c., 20 c.c. of saturated Na_2CO_3 and 2 c.c. of phenol reagent (see page 262) added. After 10, or better 30, minutes, the solution is diluted to the mark and compared in a colorimeter with a standard tryptophane solution (1 mg.; 0.1% in 5% H_2SO_4) treated similarly. For the tyrosine, to 5 c.c. of the liquid from the HgSO_4 precipitation in a 100 c.c. flask are added 30 c.c. of water, 20 c.c. of saturated Na_2CO_3 , 4 c.c. 5% NaCN , and 2 c.c. of phenol reagent, the mixture left for 10, or better 30, minutes, diluted to the mark and compared in a colorimeter with a standard made up with 1 mg. of tyrosine treated similarly.

When tryptophane is putrefied by aerobic bacteria, indole and skatole are formed. When putrefied by anaerobic bacteria, indole-propionic and indole-acetic acids are formed. The relations in the human intestine are complex, and there is evidence that both these reactions occur normally. A fraction of the bacterial derivatives are resorbed from the intestine (from the colon particularly; it is there that the bacterial reactions largely occur) and are the source of the indole substances in the urine.

Indole. C_8H_7N ; *i. e.*,



Indole is so named from being the nucleus from which the indigo group of chemical compounds is derived. It has been obtained synthetically by several reactions, and is, together with skatole, a characteristic constituent of fæces. Its occurrence therein is due to the putrefactive decomposition of tryptophane, which usually takes place to a greater or less extent in the intestinal canal, especially in the colon. The greater part of the skatole and indole formed is eliminated by the kidneys in the forms of skatoxyl- and indoxyl-sulphuric acids (the so-called "urinary indican"), the remainder being excreted in the fæces; the fractions are, however, variable.

Indole is formed when albuminous substances are fused with potassium hydroxide, but it is more easily obtained by digesting liver or fibrin with water and pancreatin. Following digestion, putrefaction is allowed to occur. If the digestion is not too prolonged, the product, when acidified and distilled, yields a distillate from which impure indole can be extracted by agitation with ether. On evaporation, the ether leaves a residue of indole mixed with skatole or phenol. From the last body indole can be purified by dissolving it in benzene and adding a benzene solution of picric acid, when indole picrate, $C_8H_7N, C_6H_3(NO_2)_3OH$, crystallises out in long red needles, which are sparingly soluble in cold, readily in hot benzene, very slightly soluble in petroleum spirit, and decomposed by ammonia.

From skatole, indole may be separated by dissolving the mixture in the smallest possible quantity of absolute alcohol, and then adding from 8 to 10 volumes of water, when the skatole will be precipitated, while the indole remains in solution.

When pure, indole forms crystalline scales of a satiny lustre. It has a persistent and disgusting fæcal odour, melts at 52° , and boils, with partial decomposition, at about 245° . Indole distils

readily in a current of steam. It is very soluble in alcohol, ether, and petroleum spirit, and dissolves with moderate facility in hot water, separating, on cooling, in oily drops, which subsequently form plates resembling benzoic acid.

Indole possesses feeble basic characters. When treated with strong hydrochloric acid it forms a sparingly soluble salt which is decomposed by boiling with water. The picrate has already been described.

If an aqueous solution of indole is treated with fuming nitric acid, or preferably with a solution of sodium nitrite acidified with sulphuric or nitric acid, a red precipitate is formed of the composition $C_{16}H_{13}(NO)N_2.HNO_3$. A deep red coloration is produced when an alcoholic solution of indole is treated with nitrous acid, or nitrogen trioxide passed through it.

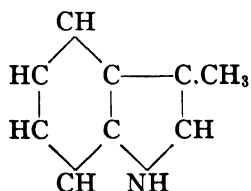
If a strip of pine wood is moistened with strong hydrochloric acid and immersed in an alcoholic solution of indole, or exposed to the vapours of indole, it is coloured deep crimson.

When a dilute solution of indole is treated with sodium nitroprusside, and a few drops of sodium hydroxide solution added, a violet-blue coloration is produced, which changes to pure blue when the liquid is acidified with acetic acid.

On melting a minute quantity of indole in a test tube with dehydrated oxalic acid, a fine magenta coloration is produced. The colouring matter formed is soluble in acetic acid. Indole, mixed with a few drops of a 5% solution of formaldehyde and strong sulphuric acid, yields a violet colour; skatole gives a yellowish-brown.

Indole is decomposed when boiled with moderately concentrated sodium hydroxide, which behaviour distinguishes it from skatole.

Skatole, or Methyl-indole,



closely resembles indole. Its odour is somewhat similar and equally persistent and unpleasant. It crystallises from hot water or,

preferably, petroleum spirit, in glittering white scales, melts at 95°, and boils at 265°.

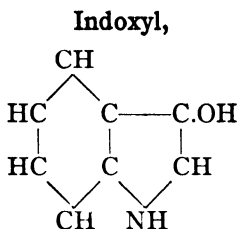
Skatole gives no colour reactions with pine wood and hydrochloric acid, nor with sodium nitroprusside, and also differs from indole in not suffering decomposition when boiled with moderately concentrated sodium hydroxide. The picrate is precipitated in red needles on mixing hot aqueous solutions of skatole and picric acid. When sodium nitrite is added to a solution of skatole in glacial acetic acid, a dark brown coloration is produced, and, on adding water, the nitrosamine is precipitated as a yellow oil, which solidifies in a freezing mixture to a crystalline mass. E. Fischer describes the formation of this compound as highly characteristic, and available for the detection of skatole and its separation from indole. When the picrates of indole and skatole are distilled with ammonia, both pass over; if they are distilled with sodium hydroxide, the skatole passes over, while the indole is decomposed.

Some additional reactions which have been proposed for distinguishing between skatole and indole may be given (V. E. Nelson, *J. Biol. Chem.*, 1916, **24**, 527). A few drops of dimethylaniline are thoroughly emulsified with 5 or 6 c.c. of skatole solution in a test tube, and concentrated sulphuric acid is added cautiously down the side of the tube so as to form a layer at the bottom. A beautiful violet ring forms at the junction of the two liquids and is still visible at a dilution of one part in 1,000,000. The substance producing this coloration is completely soluble in chloroform. Indole produces a faint red under similar conditions, but the colouring substance is not soluble in chloroform.

Pyruvic aldehyde gives with indole, sulphuric acid and a crystal of ferric sulphate, a deep red-violet colour due to substance which cannot be extracted by chloroform, carbon disulphide, or amyl acetate, but is soluble in amyl alcohol.

If to 5 or 6 c.c. of an indole solution there are added a few drops of a 5% solution of vanillin in 95% alcohol, and the mixture is made strongly acid with 3 or 4 c.c. of concentrated hydrochloric acid, a beautiful orange colouration forms. A solution of skatole to which vanillin and hydrochloric acid are added gives a deep violet colour on heating. Vanillin gives with skatole and sulphuric acid a deep red to violet colour; with indole and sulphuric acid, an orange colour. Skatole, vanillin, and phosphoric acid give a deep violet,

whilst indole under like conditions gives an orange coloration. These colour tests are not quantitative, but should be useful when taken in conjunction with the different solubilities in organic solvents of the previously described coloured substances formed.



is interesting as a substance intermediate between indole and indigotin. It possesses both basic and acid characters. Its alkaline solution absorbs atmospheric oxygen, with formation of indigo-blue, which is also produced on adding ferric chloride to a hydrochloric acid solution of indoxyl.

Indoxyl-sulphuric Acid, $\text{C}_8\text{H}_5(\text{NH}).\text{SO}_4\text{H}$, occurs in urine, largely as a potassium salt. It has received the name of "urinary indican," from a supposed identity with plant-indican, the glucoside from which indigo is obtained. The only similarity between the two substances is that both yield indigo-blue as one of the products of their decomposition.¹

Potassium indoxyl-sulphate, $\text{C}_8\text{H}_5(\text{NH}).\text{SO}_4\text{K}$, crystallises from hot alcohol in colourless lustrous tables, readily soluble in water, but only sparingly soluble in cold alcohol. When boiled with dilute acid it is decomposed into indoxyl and acid potassium sulphate, but is not attacked by alkalis. When the crystals are heated, indigotin (indigo-blue) sublimes, and the same substance is formed quantitatively when the acidified solution is warmed with ferric chloride. The quantitative determination of indoxyl and its derivatives is based upon its transformation into indigotin. A number of different oxidising agents have been proposed for this reaction, none of which is entirely satisfactory. Several of the procedures will be given.

For the detection of indoxyl-sulphuric acid in urine, Jaffé (*Pflüger's Arch.*, 3, 448) first separates any albumin by boiling the

¹ Decomposing urine occasionally forms a bluish-red pellicle, and ultimately deposits microscopic crystals of indigo-blue. A calculus of the same nature has been described.

liquid, and treats the filtrate with an equal volume of hydrochloric acid. A dilute solution of bleaching powder is then cautiously added, until the blue colour no longer increases. On agitating with chloroform the colouring matter is taken up and can be obtained on evaporation. Jaffé's method is not suitable for the detection of traces of indigogen, as the colouring matter is destroyed by the least excess of the oxidising agent. Hence MacMunn boils the urine with an equal volume of hydrochloric acid and a few drops of nitric acid, cools, and agitates with chloroform. The chloroform is generally coloured violet, and, when examined in the spectroscope, shows two broad absorption-bands, one on either side of the D line. The less refrangible is due to indigo-blue and the more refrangible to indigo-red; though it is doubtful if the latter colouring matter is identical with the indirubin which occurs in commercial indigo. Obermayer uses ferric chloride in place of the nitric acid in this test.

The chloroform extract containing the indigotin may be used for quantitative estimation by comparison in a colorimeter with a chloroform solution containing a known quantity of indigo. Titration of the indigotin as sulphuric acid derivative with potassium permanganate has also been suggested.

A. C. Méhu (*J. Pharm.*, [v], 7, 122) adds to the urine about 0.5 c.c. of strong sulphuric acid to 1 litre of the sample, and then saturates the liquid with powdered ammonium sulphate, whereby any indigotin or indirubin is precipitated. On treating the precipitate in the cold with 50% alcohol the indirubin will be dissolved, and the insoluble indigotin is then purified by washing with water, followed by spontaneous drying. Méhu proposes a colorimetric process for the estimation of indigotin, which he dissolves in hot carbolic acid to which sufficient glycerin or absolute alcohol has been added to prevent crystallisation on cooling. The colour of a solution of indigo-blue of known strength, prepared in this manner, is compared with that of the urinary pigment. Isatin reacts with indican to form two molecules of indigo-red. The reagent is prepared by dissolving 25 mg. of isatin in 100 c.c. of strong hydrochloric acid. Equal parts of this reagent and urine are simmered for a few moments, and then extracted with chloroform. Normal urine will only impart a faint pink to the layer of chloroform; abnormal urine may yield as much colour with a dilution of 1:100.

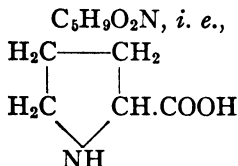
W. Michailoff (*J. Chem. Soc.*, 54, 880) also saturates the acidified urine with finely powdered ammonium sulphate, and then extracts the urobilin by repeated agitations with ethyl acetate. The aqueous layer is next mixed with an equal volume of fuming hydrochloric acid and chloroform added; it is then cautiously treated with dilute bromine-water, and well agitated after each addition. The extraction is said to be very readily effected.

The indican reaction of Jolles involves oxidation in the presence of thymol and hydrochloric acid containing ferric chloride, to form 4-thymol-2-indol-indolignone, whose hydrochloride dissolves in chloroform to give a violet colour. To carry out this test, 10 c.c. of urine are shaken with 1 c.c. of a 5% alcoholic solution of thymol. 10 c.c. of fuming hydrochloric acid, containing 5 gm. of ferric chloride per litre, are added, and the mixture carefully agitated, and allowed to stand for 15 minutes. The solution is then extracted with 4 c.c. of chloroform, which becomes intensely violet in colour. This reaction is more sensitive than the other tests described, and permits the detection of 0.0032 mg. indican in 10 c.c. of urine. Quantitative colorimetric comparison may be made with a standard solution containing 0.01 gm. of 4-thymol-2-indol-indolignone per 100 c.c. of chloroform with no hydrochloric acid present. If α -naphthol is used in place of thymol, an intense blue colour is obtained.

Indoxyl-sulphuric acid occurs in very small quantities in normal human urine, the amount being estimated as between 1 and 40 mg. per 24 hours' excretion. Horse's urine contains about 25 times as much. The proportion in human urine is much increased in certain diseases, such as cholera, typhus, peritonitis, dysentery, and Addison's disease. In obstructive diseases of the small intestine the increase is enormous. The presence of a large amount of indigogens in the urine generally implies that abundant albuminous putrefaction is in progress in some part of the system, these putrefactive products being absorbed and eliminated by the kidneys in the forms of indoxyl-sulphuric acid and its analogue skatoxyl-sulphuric acid, $C_8H_6(CH_3)N.SO_4H$. The latter substance is said to be somewhat more abundant in human urine than the indoxyl-compound. When decomposed by hydrochloric acid or an oxidising agent it gives a colouring matter, usually reddish, but which may possess a marked purple tint.

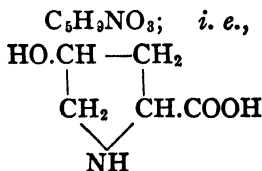
Traces of compounds of indoxyl and skatoxyl exist in normal urine, with glycuronic acid, and their proportions appear to be greatly increased under certain conditions.

Proline. α -Pyrrolidine-carboxylic acid.



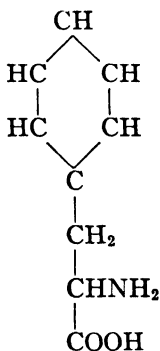
Nearly all proteins contain proline. Pyrrole is of importance in the body; mention has already been made of tryptophane, and among the other pyrroles in the body are hæmin, hæmatoporphyrin, bilirubin, urobilin and urochrome. It is not present in large amounts in the higher proteins, but in some protamines may be as much as 11%. Its isolation in the course of the ester method (see page 238), has been described. The impure material is purified by the formation of the copper salt, and the removal of the copper by hydrogen sulphide, followed by hot filtration through animal charcoal. Proline is soluble in alcohol, as well as in water, but the recrystallisation is best done from hot alcohol. The specific rotation of the naturally occurring proline is $[\alpha]_D^{20} = -77.40^\circ$. Proline is precipitated by phosphotungstic acid in sulphuric acid solution. Hydroxyproline often accompanies proline. The proline crystallises in flat needles which do not have a definite m. p. β -Naphthalene-sulpho-*l*-proline crystallises in long flat needles which melt at 138° , and are soluble in alcohol. Phenyl-iso-cyanate-proline forms resinous needles; when concentrated in 5% hydrochloric acid, the anhydride is formed, which crystallises in long flat needles, m. p. 144° . The picrate melts at $153\text{--}154^\circ$. Ethyl ester, b. p. $78\text{--}79^\circ$ at 10–11 mm, hydrolyses at ordinary temperatures in the presence of water. Ethyl ester hydrochloride is stable in water and *in vacuo* at 120° .

Hydroxyproline. γ -Hydroxy- α -pyrrolidine-carboxylic acid.



Hydroxyproline occurs in several proteins. It may be obtained from casein by long-continued digestion with trypsin. It is readily soluble in water, but can be recrystallised from a hot solution. It forms colourless plates. It is slightly soluble in alcohol. In aqueous solution hydroxyproline shows $[\alpha]_D^{20} = -76^\circ$. Its solution has a sweet taste. The phenyl-iso-cyanate compound melts at 175° ; the β -naphthalene-sulpho derivative at $91-92^\circ$. Hydroxyproline has been synthesised by Leuchs (*Ber.*, 1905, **38**, 1937).

Phenylalanine. Phenyl- α -amino-propionic acid.



This is a direct derivative of alanine and is closely related to tyrosine. It is present in all the higher or complete proteins, and seems to be most often centrally located in the nucleus of the molecule, since it is recovered late in the course of the hydrolysis of a protein. It is of great importance physiologically, though it is possible that it and tyrosine are reciprocal in the anabolic processes of the protein metabolism. The naturally occurring form rotates the plane of polarised light to the left, $[\alpha]_D^{20} = -35.7^\circ$. It is not very soluble in cold, but readily soluble in hot water, and is quite insoluble in alcohol. Phenylalanine has a bitterish taste. The crude phenylalanine, isolated as previously described, is purified by recrystallisation from hot water. The β -naphthalene-sulpho-phenylalanine crystallises in very long fine needles, m. p. 143° . The phenyl-iso-cyanate-phenylalanine forms fine colourless needles which are almost insoluble in cold water, m. p. 182° ; the picrolonate, m. p. 208° ; the picrolonate, m. p. 208° , is soluble (0.34:100) in water; and the phosphotungstate forms lemon-yellow short rhombohedral prisms.

Phenylalanine can be separated from glutamic and aspartic acids by adding enough picrolonic acid to combine with the former, when its picrolonate will crystallise out pure. Phenylalanine has been synthesised by several methods.

From tyrosine and phenylalanine are derived the phenol and cresol substances which are present in the urine. In the intestine

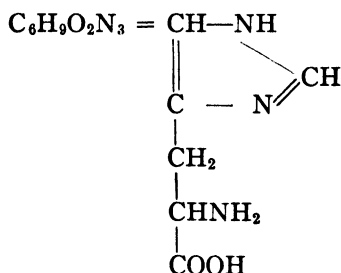


FIG. 13.—Phenylalanine. $\times 100$. (From Keenan, *J. Biol. Chem.*, 1924, 62, 163.)

these two amino-acids are subjected to bacterial putrefaction, and as the reactions are not completed, we have in the urine several aromatic substances which are derived from this intestinal decomposition. The list of aromatic substances includes the following. Phenol-sulphuric acid; cresol-sulphuric acid; pyrocatechin and quinol, combined with sulphuric acid; *p*-hydroxy-phenyl-lactic acid and *p*-hydroxy-phenyl-propionic acid; and probably dihydroxy-phenyl-lactic acid. In these conjugations of the aromatic substances the sulphuric acid may be substituted by glucuronic acid. In the metabolic anomaly known as alkaptonuria, we have in the urine

an abnormal aromatic body, homogentisic acid (dihydroxy-phenyl-lactic acid), supposed to be a normal intermediary stage, which appears as an end-product in this condition.

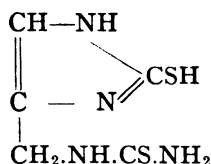
Histidine. α -Amino- β -iminazolypropionic acid.



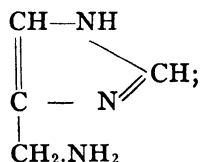
Histidine is contained in nearly all complete proteins, and in some of the simpler protamines. Globin is especially rich in histidine. Histidine is a base, very soluble in water, quite soluble in alcohol. The aqueous solution rotates the plane of polarised light to the left, $[\alpha]_D^{20} = -39.74^\circ$; dissolved in hydrochloric acid, the solution is dextrorotatory. It is purified by precipitation by the addition of a solution of mercuric sulphate acidified with sulphuric acid, the precipitate washed, the mercury removed by hydrogen sulphide, the filtrate freed from sulphuric acid by barium hydroxide, the excess of barium removed with carbon dioxide, the final filtrate concentrated, and hydrochloric acid added until the reaction is faintly acid. On concentrating and standing, the hydrochloride will crystallise out in large plates. The presence of alcohol aids the crystallisation. When histidine is heated with hydrochloric acid in methyl alcohol, the methyl ester is formed, the dihydrochloride of which is a crystalline salt, m. p. 196° . Histidine responds to the biuret test. On warming with bromine water, a red coloration gradually appears, followed by the deposition of reddish particles. The colour is destroyed by excess of bromine, but the coloured compound is soluble in chloroform. The maximum colour is obtained with 3 atoms of bromine to one molecule of histidine hydrochloride. The test is sensitive to 1 part in 10000. If a solution of histidine is made alkaline with carbonate, the addition of diazo-benzene-sulphonic acid will cause the production of an intense red diazo reaction. Tyrosine gives a similar colour. If the coloured

compound is reduced by zinc in hydrochloric acid solution, and then made alkaline with excess of ammonia, histidine gives a golden yellow colour, tyrosine a rose red. This reaction is specific for histidine and is given in concentrations greater than 1:20,000, but is not specific for tyrosine, although it is useful. The most characteristic salt is the picrolonate. Picrolonic acid in alcoholic solution is added to the solution of histidine, in slight excess only, and the mixture set aside for several days for crystallisation. The crystals are washed with cold water, and may be dried and weighed for a quantitative estimation. The fine yellow needles melt at 220° . The phosphotungstate forms large, clear rhombohedral prisms.

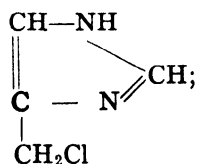
Histidine has been synthesised by several methods. The most interesting method was given by Pyman (*J. Chem. Soc.*, 1911, **99**, 672, 1392, 2172; 1916, **109**, 186) and consisted of the following transformations:—Citric acid; acetone dicarboxylic acid; di-isonitroso acetone; diaminoacetone; iminazole compound



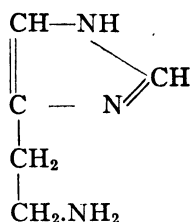
(action of potassium thiocyanate); (action of nitric acid)



(nitrous acid and phosphorus pentachloride)



(sodium chlormalonic ester, hydrolysis, removal of carbon dioxide and treatment with ammonia) inactive histidine; (fractional crystallisation with tartaric acid) *l*-histidine.

Histamine (β -Iminazolethylamine) $C_6H_9N_3$; *i. e.*,

Histamine is a highly potent substance which has been found in extracts of a number of glands. It may be an important factor in their actions, and, in fact, for a time, was considered to be the active principle in extracts of the posterior lobe of the hypophysis. It is present in the bacterial or putrefactive decomposition of proteins. It is one of the active principles of ergot. It may be formed by bacterial growth (certain strains of *B. coli* are most satisfactory, M. T. Hanke and K. K. Koessler, *J. Biol. Chem.*, 1922, **50**, 131) on synthetic media containing 0.1% added histidine. Histamine may be isolated from such a mixture, after removal of the ammonia, by precipitation with picric acid and recrystallisation of the picrate. From more complex mixtures, such as ergot or putrefying meat, it is necessary to fractionate first with silver nitrate and barium hydroxide, histamine remaining with the histidine fraction. The hydrochloride is separated from inorganic salts by extraction with methyl alcohol.

Histamine dihydrochloride is easily soluble in water, sparingly soluble in ethyl alcohol. It forms prisms, m. p. 240° . The chloroplatinate forms orange-coloured prisms soluble in water, but only slightly soluble in alcohol. The dipicrate forms deep yellow rhombic leaflets, m. p. $238-242^\circ$, and can be recrystallised readily from water. Histamine can be isolated most readily from mixtures by means of this salt. The monopicrate crystallises in the form of pointed needles, m. p. $233-234^\circ$. The dipicrolonate, soluble in 450 parts of boiling water, forms needles, m. p. about 264° . The dinitrate forms six-sided prisms, m. p. $149-150^\circ$. By shaking histamine with benzoyl chloride in chloroform for ten hours, the benzoyl derivative (side-chain substitution product) is formed, six-sided prisms and plates, m. p. 148° . It may be crystallised from ether or petroleum

spirit, and may be used for isolating histamine from putrefactive mixtures.

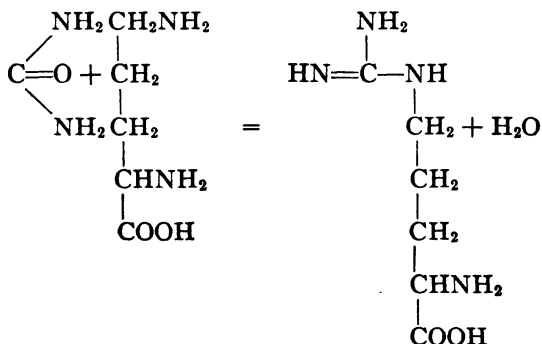
Histamine gives many of the reactions of histidine, but may be distinguished from it by not giving the biuret reaction, nor a colour with triketohydrindene hydrate.

Histamine may be obtained in a yield of 30% of the theoretical amount by heating histidine with hydrochloric or sulphuric acid in a sealed tube at 265–270°. It has been synthesised by Pyman.

The physiological actions of histamine have been studied extensively in recent years, because of its possible presence in certain glands and under various putrefactive conditions. The more recent work indicates that, although histamine is not the active constituent of the pituitary gland, it may be formed at times because of putrefactive actions there and elsewhere. Its physiological action, when injected into a guinea pig, is strikingly suggestive of anaphylactic shock. It has also been suggested that it plays an important rôle as stimulant for the gastric and intestinal musculature, and also as a dilator of capillaries during digestion.

The occurrence of histamine in natural products and its probable rôle in various physiological and pathological processes is still under active investigation.

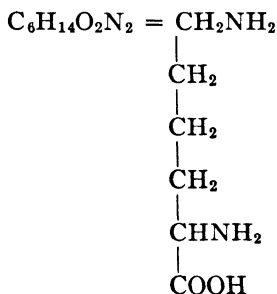
Arginine (Guanidine- α -amino-valeric acid).—This amino-acid is a combination of urea with ornithine, according to the equation:



Arginine is a component of nearly all proteins, the simple protamines being especially rich in it. It is strongly basic, and very soluble in water, slightly soluble also in alcohol. It is dextro-rotatory, $[\alpha]_D^{20} = +21.25^\circ$ in hydrochloric acid solution. It is decomposed by a special enzyme of the liver and kidney, arginase,

with the production of urea and ornithine. It may be prepared synthetically from ornithine and cyanamide. It forms both simple and double salts. The combinations with silver (used for its isolation), and with copper nitrate, are quite insoluble. The β -naphthalene-sulpho-arginine forms light powdery crystals, the m. p. being 87° . Dibenzoyl-arginine crystallises in long plate-like needles which melt at 218° . The phosphotungstate forms a white amorphous powder, or, after recrystallisation, large rhombohedral crystals. There is a picrate, but the most characteristic salt is the picrolonate, which forms yellow needles which melt at 110° , and are very insoluble in both water and alcohol. A quantitative estimation is best made by weighing the picrolonate. It is stated that arginine forms an almost insoluble salt with "naphthol yellow S" (2,3-dinitro-1-naphthol-7-sulphonic acid), easily prepared, isolated, and estimated (Kossel and Gross).

Lysine. α - ϵ -Diamino-caproic acid.



Lysine is a base widely present in both animal and plant proteins. It is very soluble in water, and does not crystallise as such. The naturally occurring form is dextrorotatory, $[\alpha]_D^{20} = 14^{\circ}$ to 17° , depending upon the relations of concentration. Heating with barium hydroxide converts it into the racemic state. Lysine is subject to bacterial putrefaction and is the parent substance of penta-methylene-diamine. It forms acid and double salts, *i. e.*, with silver nitrate one salt will have included a molecule of nitric acid, the other not. The lysine, isolated as previously described (see page 242), is best purified by precipitation with picric acid. To the solution of lysine, concentrated almost to dryness, an alcoholic solution of picric acid is added so long as a precipitate forms. After a couple of days the crystals of picrate are filtered

off, washed with absolute alcohol, dissolved in a small amount of boiling water, filtered, and the filtrate concentrated, following which the crystallisation will again occur on cooling. The crystals may then be washed in absolute alcohol and, when weighed, give a fair result for a quantitative estimation. To recover the lysine, the crystals of picrate are dissolved in hot 5% sulphuric acid, removed by shaking with ether, and the lysine precipitated with phosphotungstic acid as previously described, and again isolated as before. The phosphotungstate can be recrystallised and forms fine needle-shaped crystals. The phenyl-iso-cyanate forms, when boiled with hydrochloric acid, a hydantoin which melts at 183° .

QUANTITATIVE ESTIMATION OF MONOAMINO-ACIDS

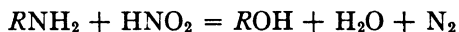
This may now be accomplished in one of two ways. If the total amino-acids are to be estimated, the histidine, lysine and arginine may be separated as previously described and estimated by the Kjeldahl method. For the estimation of the monoamino-acids, the material (urine, culture medium, tissue extract, etc.) is suspended in hot water and filtered. The cooled filtrate is acidified with sulphuric acid to a 5% concentration, and then precipitated with phosphotungstic acid (10% solution in 5% sulphuric acid). The precipitate is collected upon a Buchner funnel, thoroughly washed with 5% sulphuric acid, the excess of the reagent and the sulphuric acid removed with barium hydroxide, and the excess of the barium removed by the careful addition of sulphuric acid or of carbon dioxide. In the final filtrate are the amino-acids.

The method of Sørensen is based upon the previously stated fact that formaldehyde combines with the NH_2 groups of amino-acids, and from the estimation of the carboxyl groups, the quantity of the amino-acids may be calculated. Phenolphthalein (or thymolphthalein) is used as indicator and $N/5$ barium or sodium hydroxide solution for titration. The original solution containing amino-acid (or similar compound) is brought to a point neutral to litmus with the alkali, neutralised formaldehyde solution added, and the solution titrated to a definite and fairly deep colour (phenolphthalein or thymolphthalein). The second titration value, less the amount required to bring water to the same colour under the same conditions, gives the number of carboxyl groups set free corresponding to the amino groups removed by the formaldehyde. Each c.c. of $N/5$

alkali corresponds to 2.8 mg. of NH_2 nitrogen. Any ammonia present is similarly removed by the formaldehyde. A convenient method for the formol titration was described by J. H. Northrop (*J. Gen. Physiol.*, 1925-26, **9**, 767).

The estimation of amino-acid nitrogen in fluids such as urine by this method has been modified by using phosphotungstic acid to remove ammonia and other impurities (S. R. Benedict and J. R. Murlin, *J. Biol. Chem.*, 1913, **16**, 385). The method will be given as illustrating a practical application of the reaction:—200 c.c. of urine (24 hours' specimen diluted to 2,000 c.c.) are measured into a 500 c.c. Erlenmeyer flask, an equal quantity of 10% phosphotungstic acid (Merck's) in 2% hydrochloric acid added, allowed to stand at least 3 hours, 250 c.c. clear liquid decanted, 1 c.c. of a 0.5% phenolphthalein solution added, and then barium hydroxide, a little at a time, until the whole fluid turns decidedly pink. The mixture is allowed to stand 1 hour, two 100 c.c. samples (= 50 c.c. urine) are filtered off, neutralised to litmus with $N/5$ hydrochloric acid, 16-20 c.c. of neutralised formaldehyde solution added, and titrated to a deep red colour. The result is corrected by deducting the amount of $N/10$ sodium hydroxide necessary to produce the same depth of colour in an equal quantity of water, freed from carbon dioxide, to which the same quantity of neutral formaldehyde has been added.

The method of estimation of Van Skyke utilises the reaction of amino-nitrogen with nitrite as follows:



By the measurement of the nitrogen, the estimation may be very accurately accomplished. Since nearly all the natural materials that one might desire to submit to such an analysis contain either urea or ammonia or both, these must first be removed. The urea is decomposed by soya bean urease, as described in a later section (page 346). This converts the urea into ammonia, but does not affect the amino-acids. The solution is then transferred to a distillation flask, made alkaline with an excess of calcium hydroxide and distilled until the fumes are free from ammonia. The foaming may be moderated by the addition of a piece of paraffin wax or a little caprylic alcohol. The residue is then filtered, the precipitate on the filter carefully washed with hot water, and the combined filtrate then

evaporated nearly to dryness. The final solution is then filtered into a 25 c.c. flask, the filter paper repeatedly washed with small portions of water, and the flask filled up to the mark. The final solution should be made acid with sulphuric acid, and for the estimation an amount should be taken containing not over 20 mg. of amino-nitrogen.

The form of apparatus has been modified and improved in various particulars by Van Slyke (*J. Biol. Chem.*, 1911, **9**, 186; 1912, **12**, 275; 1913, **16**, 122). The method and procedure can be given best in his own words.

Principle of the Method.—Nitrous acid in solution spontaneously decomposes, with formation of nitric oxide:



This reaction is utilised in displacing all the air in the apparatus with nitric oxide. The amino solution is then introduced, evolution of nitrogen mixed with nitric oxide resulting. The oxide is absorbed by alkaline permanganate solution, and the pure nitrogen measured in the special gas burette shown in the figure.

Reagents.—The permanganate as absorbent for nitric oxide was chosen after trial of all the solutions recommended in the literature. Ferrous sulphate solution, which is ordinarily recommended in gas analysis methods, is entirely unsatisfactory. The reaction by which ferrous sulphate and nitric oxide combine is reversible, and the nitric oxide in solution attains an equilibrium with that in the supernatant gas. Therefore even approximately complete absorption is possible only with perfectly fresh ferrous sulphate solution, and even with this, is a comparatively slow process. Results become inaccurate before the solution has absorbed its own volume of nitric oxide. Sulphite solution, recommended by Divers, is even less satisfactory. A strong solution of sodium dichromate in sulphuric acid, which oxidises the oxide to nitric acid, is better, but is somewhat viscous. Acid permanganate, unless in very dilute solution, gradually decomposes, giving off oxygen, which super-saturates the solution. 1% permanganate in 1% sulphuric acid gives accurate results, however, if the solution is freed from excess oxygen by shaking thoroughly with air immediately before use. Alkaline permanganate, originally employed by Hans Meyer, proved an absolutely satisfactory absorbent solution in every

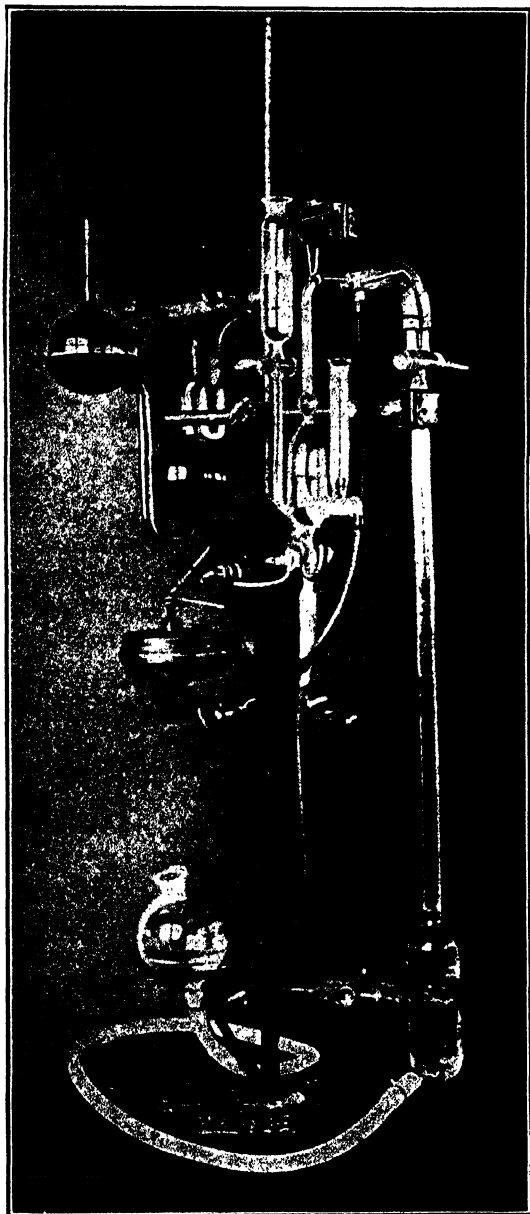


FIG. 14.—Van Slyke's amino nitrogen apparatus. Complete apparatus assembled for permanent use.

respect. It is entirely stable, can be used in concentrated solution, and oxidises the nitric oxide to nitrate with such rapidity that the gas is absorbed about as fast as is carbon dioxide by potassium hydroxide solution. A solution containing 50 grm. of potassium permanganate and 25 grm. of potassium hydroxide per litre was adopted for permanent use. The manganese dioxide formed by reduction is in such a fine state of division that it does not interfere at all with the use of the solution in a Hempel absorption pipette, and a large number of determinations can be made without changing the solution. In order to prevent deposition of manganese dioxide in the capillaries, it is well to leave G (see Fig. 14) filled with water from the gas burette, rather than with permanganate, when the apparatus is not in use. As the alkaline solution absorbs carbon dioxide as well as nitrogen, the presence of carbonate in the amino solution does not interfere with the determination.

For decomposing the amino substance the most satisfactory conditions are, to have a great excess of nitrite, from which the nitrous acid is freed by an equivalent of a weak acid (acetic). The great excess of reagent forces the reaction to rapid completion. The use of a weak acid, instead of the mineral acids employed in previous methods, causes evolution of a relatively small volume of nitric oxide, and avoids danger of acid hydrolysis of the more complex proteolytic products. In dissolving amino substances not readily soluble in water alone, one may use mineral acids of not more than $N/2$ concentration, acetic acid of any concentration up to 50%, or fixed alkali up to $N/1$ concentration. A few drops of sodium hydroxide solution are usually added to assist in dissolving tyrosine and lysine picrate.

Corrections for Impurity in Reagents.—As commercial sodium nitrite often contains impurities which gradually evolve traces of nitrogen when the nitrite is acidified, each lot of the latter must be tested before it is used, and, a correction for the reagent employed, if necessary, in calculating subsequent results. A typical "C.P." commercial nitrite yielded 0.2 c.c. of nitrogen in 5 minutes, 0.3 c.c. in $\frac{1}{2}$ hour, and 0.5 c.c. in 2 hours.

Apparatus.—The apparatus which was described first is the larger form.¹ Van Slyke later devised a micro form which answers

¹ The apparatus may be obtained from Emil Greiner, 45 Cliff St., New York City; from Robert Goetze (Leipzig); and in England from Müller, Orme & Co.

most requirements and which will be described briefly, following the description of the larger form.

The reaction is carried out in *D*, a bulb of 40–45 c.c. capacity. *A*, of about 35 c.c. capacity, serves to hold water which is used to displace air from *D*, or to receive solution forced back from *D* by nitric oxide. The 10 c.c. burette, *B*, holds the solution of amino substance for analysis. The wire from which the deaminising bulb, *D* is suspended should be fairly stiff, and rigidly fastened in position from above, so that the loop about the capillary acts as a fixed centre. *A* is then so placed that its centre of gravity comes near this centre, and the shaking of *D* is accomplished with a minimum motion in *A* and, consequently, without putting a dangerous strain on the tube which connects *A* and *D*. This tube is strong-walled and of 3 mm. inner diameter. The reason for this is that during the analysis gas containing some nitrogen collects in the tube. Unless *a* is of as wide bore as the tube, the liquid from *A* may flow around the bubble instead of forcing it into *D* at the end of the reaction. The cock is also of large bore in order to facilitate emptying *D*. The neck connecting *D* and *B* must be of at least 8 mm. inner diameter in order to allow free circulation of the solution in *D* up to the cock, *B*. The small bulb at the top of *D* keeps the reacting solution from splashing into the capillary.

In order to insure tightness of the cocks and to prevent their becoming loosened by vigorous shaking, it is well to lubricate them with a paste made by dissolving together over a flame one part of rubber, one part of paraffin and two parts of vaseline.

A determination consists essentially of three parts.

1. *Displacement of Air by Nitric Oxide.*—The capillary leading to the Hempel pipette and also the other capillary as far as *c* is filled with water from *F*. Into *A* is poured a volume of glacial acetic acid sufficient to fill one-fifth of *D*. For convenience, *A* is etched with a mark to measure this amount. The acid is run into *D*, cock *c* being turned so as to let the air escape from *D*. Through *A* is now poured sodium nitrite solution (30 gm. NaNO_2 to 100 c.c. H_2O) until *D* is full of solution and sufficient excess is present to rise a little above the cock into *A*. It is convenient to mark *A* for measuring off this amount also. The gas exit from *D* is now closed at *c*, and, *a* being open, *D* is shaken for a few seconds. The nitric oxide, which instantly collects, is let out at *c*, and the shaking

repeated. The second lot of nitric oxide, which washes out the last portions of air, is also let out at *c*. *D* is now connected with the motor and shaken till all but 20 c.c. of the solution have been displaced by nitric oxide and driven back into *A*. A mark on *D*

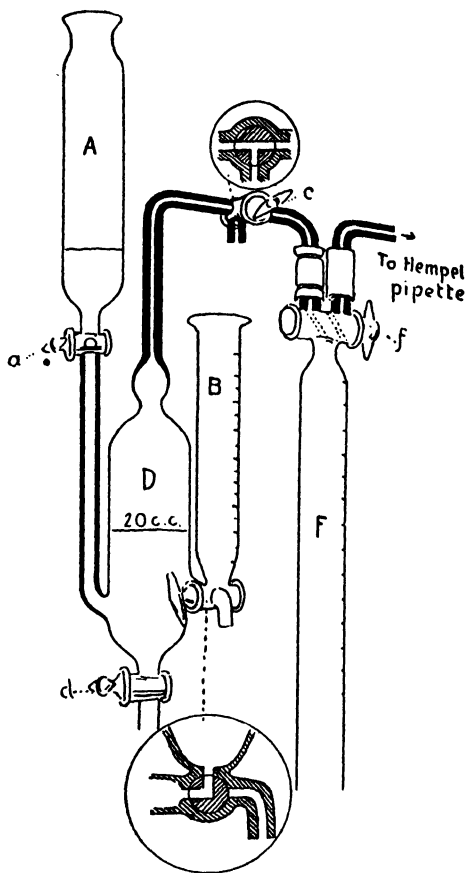


FIG. 15.—Deaminising bulb and connections in detail.

indicates the 20 c.c. point. One then closes *a* and turns *c* and *f*, so that *D* and *F* are connected. The above manipulations require between one and two minutes.

2. *Decomposition of the Amino Substance.*—Of the amino solution to be analysed, 10 c.c. or less, as the case may be, are measured off in *B*. Any excess added above the mark can be run off through

the outflow tube. The desired amount is then run into *D*, which is already connected with the motor, as shown in the figure. It is shaken, when α -amino-acids are being analysed, for a period of three to five minutes. With α -amino-acids, proteins or partly or completely hydrolysed proteins, we find that at the most five minutes' vigorous shaking completes the reaction. (Only 95% of the lysine nitrogen reacts in five minutes, but the remaining one-twentieth of the lysine nitrogen is a practically negligible proportion of the total nitrogen of a complete protein.) Only in the cases of some native proteins which, when deaminised, form unwieldy coagula that mechanically interfere with the thorough agitation of the mixture, a longer time may be required. In case a viscous solution is being analysed and the liquid threatens to foam over into *F*, *B* is rinsed out and a little caprylic alcohol is added through it. For amino substances, such as amino-purines, requiring a longer time than five minutes to react, the reacting solutions are merely mixed and allowed to stand for the required length of time, and then shaken for about two minutes to drive the nitrogen completely out of solution.

When it is known that the solution to be analysed is likely to foam violently, it is advisable to add caprylic alcohol through *B* before the amino solution. *B* is then rinsed with alcohol and dried with ether or a roll of filter paper before it receives the amino solution.

3. *Absorption of Nitric Oxide and Measurement of Nitrogen.*—The reaction being completed, all the gas in *D* is displaced into *F* by liquid from *A*, and the mixture of nitrogen and nitric oxide is driven from *F* into the absorption pipette. The driving rod is connected with the pipette by lifting the hook from the shoulder of *d* and placing the other hook, on the opposite side of the driving rod, over the horizontal lower tube of the pipette. The latter is then shaken by the motor for a minute, which, with any but almost completely exhausted permanganate solutions, completes the absorption of nitric oxide. The pure nitrogen is measured in *F*. During the above operations *a* is left open, to permit displacement of liquid from *D* as nitric oxide forms in *D*.

Testing Completeness of Reaction.—As a rule, there is little danger of failing to obtain a complete evolution of nitrogen. The point may be tested, however, as follows:—The nitrogen from *F* is driven

out at *c*; *a* is closed and *D* connected with *F*. The gas which has formed in the nitrous acid solution in *D* during the absorption of the nitric oxide and measurement of nitrogen is shaken out and driven over into *F* and then into the Hempel pipette, as before. After the gas has all been forced from *D* over into *F* at the end of the reaction, the nitrous acid solution is run out from *D*, by opening *d*, through a tube leading to a drain. *B* is rinsed and dried with a roll of filter paper or with alcohol and ether, and the apparatus is immediately ready for use again.

Blank determinations, made as above, except that 10 c.c. of distilled water replace the solution of amino substances, must be made on every fresh lot of nitrite used. The amount of gas obtained on a five-minute blank is usually 0.3 to 0.4 c.c., with very little increase for longer tests. Nitrite giving a much larger correction should be rejected.

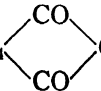
Amino groups in the α -position to the carboxyl, as in the neutral amino-acids, react quantitatively in 5 minutes at 20°. The group in lysine requires 30 minutes to react completely, lysine being the only natural amino-acid which requires more than five minutes. Ammonia and methylamine require 1.5 to 2 hours to react quantitatively. Urea requires 8 hours. Amino groups in purines and pyrimidines require 2 to 5 hours at 20°. For these substances, the procedure is modified as already stated.

The micro form of apparatus devised by van Slyke (*J. Biol. Chem.*, 1913, **16**, 122) answers all the requirements of ordinary work. The gas burette in this form holds 10 c.c., the upper part measuring the first 2 c.c. is 4 mm. in diameter and is divided into divisions of 0.02 c.c.; the remainder is wider and divided into 0.05 c.c.; the deaminising bulb has a volume of 11 to 12 c.c., and the burette of 2 c.c. of nitrite solution, and 2.5 c.c. of acetic acid are required for each analysis and the correction for the reagents is 0.06–0.12 c.c. Only 0.5 mg. of amino nitrogen is required for an analysis accurate to within 1%. The manipulations are the same as with the larger apparatus, except that in the first stage of the analysis in freeing the apparatus from air the deaminising bulb should be shaken by the motor at a very high speed—about as fast as the eye can follow. In the third stage, when the nitric oxide is absorbed by the permanganate, the Hempel pipette should be shaken not faster than twice per second.

A useful table for the conversion of c.c. of nitrogen gas into mg. of amino-nitrogen at various temperatures and pressures was given by van Slyke (*S. Biol. Chem.*, 1912, **12**, 275). A table extending these values to include pressures from 530 to 780 mm. and temperatures between 16° and 30° was published recently by P. F. Sharp (*S. Biol. Chem.*, 1924, **60**, 77).

A test, which may be mentioned here, is that devised by O. Folin for the estimation of amino-acids in blood and in urine (*J. Biol. Chem.*, 1922, **51**, 377, 393). It consists essentially in the development of a red colour by the interaction of amino-acids with β -naphthoquinonesulphonate, and the destruction of the excess of the reagent with sodium thiosulphate. The reaction is not given by urea, uric acid, creatine, creatinine, or hippuric acid, but is given by ammonia, which must therefore be removed, preferably by permute.

A new amino-acid containing sulphur has been isolated first from casein, then also from egg albumin, edestin, and wool (J. H. Mueller, *J. Biol. Chem.*, 1923, **56**, 157). It is present in amounts of 0.2 to 0.4%. The analysis and molecular weight determinations indicate the formula $C_5H_{11}NO_2S$. It was shown by Barger and Coyne (*Biochem J.*, 1928, **22**, 1417) to be λ -methylthiolbutyric acid, possessing the structure $CH_3S.CH_2.CH(NH_2).CO_2H$. The name methionine was given it. It melts, with decomposition, at 283°, is easily soluble in cold water, in which it shows the rotation $[\alpha]_D^{20} = -7.2^\circ$, and forms white, hexagonal plates, often massed together. The α -naphthol isocyanate compound melts at 186°. The amino-acid is best obtained by hydrolysis of the proteins by sulphuric acid, but has also been obtained in the alkaline hydrolysis of casein. It is separated by repeated precipitation as the mercuric salt, and solution by barium hydroxide, treatment with silver oxide, and final precipitation with alcohol. The substance can be purified by re-precipitation with mercury salts.

Ninhydrin Test.—The conditions for carrying out the triketohydrindene hydrate, C_6H_4  $C(OH)_2$, (*ninhydrin*¹) test for the presence of amino-acids, peptides, peptones, proteins, etc.,

¹ Put on the market by Meister Lucius and Brüning, Höchst a.M.

have been studied in detail.¹ In carrying out this test, 2-10 c.c. of the solution in question are boiled for 1 minute with 0.2 c.c. of a 1% aqueous triketohydrindene hydrate solution. The appearance of a blue colour shows the presence of a substance containing a carboxyl and an amino-group attached to an aliphatic radical. Certain precautions are necessary. The solutions must be neutral. A blue coloration will appear if a strongly alkaline ninhydrin solution is warmed, but this colour disappears on dilution. Since the coloured substance obtained is colloidal and sensitive towards electrolytes (cations exerting the predominating action), only small amounts of neutral salts may be present. The test is given most readily if the amino-group is in the α position to the carboxyl group; if it is in the β , γ , δ , ϵ , positions, the blue colour appears only on heating. Fairly concentrated solutions of alcohols, ketones, and reducing sugars give a red or blue coloration when warmed with a ninhydrin solution; the colour is intensified by the addition of alkali and is said to be different from that obtained with amino-acids, etc. Addition of alkali to the mixture after the colour has developed, does not dissipate the colour. The reagent is sensitive for the following substances in the dilutions indicated: Glycocoll, 1:65,000; *D*-alanine, 1:26,000; *D*-valine, 1:15,000; *L*-leucine, 1:25,000; *D*-glutamic acid, 1:22,000; asparagine, 1:19,000; *DL*-phenylalanine, 1:26,000; *L*-histidine, 1:79,000; α -aminobutyric acid, 1:16,000.

It is very probable that practically all of the amino-acids present in proteins to any extent have been identified, although from time to time small amounts of new amino-acids may be found. The quantitative estimation of many of these constituents is, however, not so certain. The following table gives a list of the constituent amino-acids of a number of proteins. The list is not complete in any way, so far as the number of proteins which have been analysed is concerned. The amounts given have been compiled from the best data available at the present time, and the accuracy of the determinations for the different amino-acids in any one protein must be looked upon as of variable reliability. Some of the estimations are fairly accurate, others are unquestionably low, and others may refer to mixtures. Thus it is probable that some of the

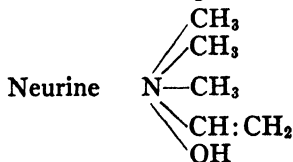
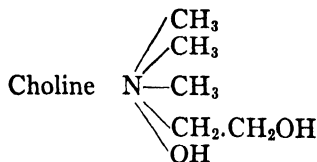
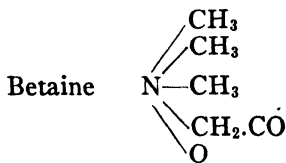
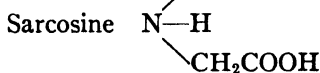
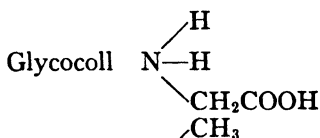
¹ Paul E. Howe, *Biochem. Bulletin*, 1914, 3, 269; Abderhalden and Schmidt, *Z. physiol. Chem.*, 85, 143; E. Herzfeld, *Biochem. Z.*, 59, 249; Halle, Löwenstein and Pribram, *Ibid.*, 55, 357.

	Glycocoll	Alanine	Leucine	Isoleucine	Valine	Glutamic acid	Hydroxy glutamic acid	Aspartic acid	Phenylalanine	Tyrosine	Cystine	Serine	Proline	Hydroxy proline	Tryptophane	Histidine	Arginine	Lysine	Ammonia	Amino-valeric acid
Serum albumin.....	0	2.7	20.0	7.7	3.1	3.1	5.7	4.2	0.6	1.0	1.4	3.4	4.9	13.2
Serum globulin.....	3.5	2.2	18.7	8.5	2.5	3.8	6.6	2.25	3.0	4.0
Lactalbumin.....	0.37	2.41	10.4	3.30	10.1	10.0	0.30	2.4	0.9	1.7	1.76	3.76	3.1	2.6	3.7	10.3	1.5
Gladiin (wheat).....	0.68	2.0	6.62	3.34	43.66	0.58	2.35	3.1	2.40	0.13	13.22	1.68	2.19	2.97	1.21	5.22
Glutenin.....	4.56	1.8
Edestin (hemp used).....	3.8	3.6	20.9	5.6	18.74	4.25	3.1	4.5	0.97	0.33	4.1	2.0	1.5	1.1	11.7	1.0	1.88
Vitelin.....	1.1	0.75	11.0	2.4	12.95	2.13	2.8	3.37	4.18	1.74	1.9	7.46	4.81	1.25
Vetch legumin.....	0.38	2.08	8.0	1.0	13.8	5.3	3.75	1.55	0.53	3.22	1.05	2.42	10.12	4.29	1.75
Zein.....	0	9.79	25.0	1.88	31.3	2.5	1.71	7.6	5.9	0.75	1.02	9.04	0.17	0.82	1.8	0	3.61
Gelatin.....	25.5	8.7	7.1	0	0	5.8	0	3.4	1.4	0	0.2	0.4	9.5	14.1	0	0.9	8.2	5.9	0.4
Casein.....	0	1.5	9.35	1.43	7.20	21.0	10.5	1.30	3.20	6.37	0.26	0.5	6.70	0.23	1.4	3.8	4.53	7.7	1.60
Fibrin.....	3.0	3.6	15.0	1.0	10.4	2.0	2.5	4.4	3.72	0.8	3.6	2.0	6.4	7.4	11.1
Wool.....	5.52	7.8	1.45	6.0
Keratin (horn).....	0.34	1.20	18.3	5.79	3.0	2.50	3.00	5.28	6.07	0.68	3.60	1.43
Egg albumin.....	0	2.2	10.7	2.5	9.1	2.2	5.2	4.0	0.88	3.6	1.3	1.7	4.9	3.8	1.3
Hordein.....	5.03	1.67	1.18	13.73	2.27	2.82	0.89	4.87
Scombrin (Protamine, mackerel).....	0	0.43	5.67	0.13	43.19	3.8	88.8
Salmin (Protamine, salmon).....	6.8	3.2	4.3	8.74	1.6

older determinations of leucine included also variable amounts of isoleucine. With these limitations, the accompanying table may be of interest and of possible value.

Betaines

Glycocoll may be considered to be the starting point of two series of bases. The one series, having the characteristics of amino-acids, contains the amino group intact, substituting groups entering on the hydrocarbon nucleus. These are the amino-acids which have just been considered. The second series, which will now be considered, contains hydrocarbon or similar groups in place of the hydrogen atoms of the amino grouping at the same time that the remainder of the molecule may be modified as with the simpler amino-acids. The compounds included under this heading are generally termed *Betaines*. These occur abundantly in vegetable products, but are also obtained at times from animal sources. Some of the latter will now be considered. The following list shows the structural relationship of these compounds.

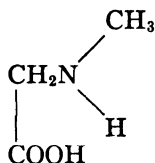


It is evident that the structural formulae for glycocoll and sarcosine may also be written in the anhydride form and would then show

greater analogies to betaine. There is no certain evidence as to which structures are ordinarily to be assigned to these two compounds.

Sarcosine. Methyl-glycocoll

$C_3H_7O_2N$; *i. e.*,



Sarcosine is prepared by boiling creatine with an aqueous solution of 10 times its weight of barium hydroxide until all odour of ammonia has disappeared. The creatine is decomposed into sarcosine and urea, the latter product being further split up into ammonia and carbonic acid. The excess of barium hydroxide is removed by a current of carbon dioxide, the liquid boiled, filtered, and evaporated to a syrup, from which the sarcosine is deposited in foliated crystals on standing. Sarcosine also results from the action of acids or alkalies on caffeine and theobromine (Vol. VII); and W. Paulmann (*Arch. Pharm.*, **232**, 601) recommends the hydrolysis of caffeine as the best method for the preparations of sarcosine, the yield being 60% of the theoretical.

Sarcosine may be purified by conversion into the sulphate, the aqueous solution of which is then decomposed by pure barium carbonate.

Sarcosine crystallises in colourless, transparent, rhombic prisms, having a sharp, sweetish, somewhat metallic taste. Sarcosine is unchanged at 100° , but at a higher temperature melts and volatilises without leaving any residue.

Sarcosine is readily soluble in water, sparingly soluble in alcohol, and insoluble in ether. It combines with acids to form soluble crystallisable salts. $B_2, H_2SO_4 + 2H_2O$ forms colourless, quadrangular crystals, very readily soluble in water. $B_2, H_2PtCl_6 + 2H_2O$ is soluble in water, and crystallises in large, pale yellow, flattened octahedra. Sarcosine also reacts with bases. The cupric salt, $Cu(C_3H_6NO_2)_2 + 2H_2O$, forms deep blue crystals.

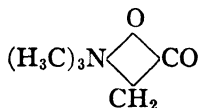
When heated with soda-lime, sarcosine evolves methylamine.

Sarcosine is isomeric with alanine, lactamide, and urethane. It is distinguished from these substances by its insolubility in alcohol and ether, in addition, of course, to various chemical reactions.

Benzoyl-sarcosine (methyl-hippuric acid), NMeBz.CH.COOH , has been prepared, but owing to its extreme solubility has not been obtained in crystals.

In all its chemical relationships sarcosine presents a close resemblance to glycocoll.

Betaine. Dimethyl-sarcosine. Hydroxycholine



Betaine is usually regarded as having the constitution of an internal anhydride.

Betaine occurs naturally in the juice of beetroot (*Beta vulgaris*). The unripe root contains 0.25, but the ripe root only 0.10%. The betaine is not present in the root as such, but in a form from which it may be liberated by treatment with hydrochloric acid or barium hydroxide. Betaine is also present in beetroot molasses, in the branches and leaves of *Lycium barbarum* (whence its name lycine), in mangold wurzel, in cotton-seed, etc., and is a product of the decomposition of proteins.

A number of methods have been given for preparing betaine from molasses or beetroot juice. Perhaps the most satisfactory is to extract the base from the desaccharified residue by 96% alcohol, evaporate the alcohol, and convert the base into the hydrochloride, which is purified by recrystallisation.

A different process was described by Stoltzenberg (*Ber.*, 1912, 45, 2248). This depends upon the changes in solubility produced by saturating solutions containing betaine from molasses residues with hydrochloric acid; the solubility of potassium chloride decreases from 34 to 1.9 parts per 100 at 20°, that of glutamic acid hydrochloride from 38 to 9.4, whilst that of betaine hydrochloride increases from 40 to 41 parts. In a mixture of the three, the latter has a "salting-out" effect on the other two.

Betaine and its isomeride, the methyl ester of dimethyl-amino-acetic acid, $(\text{CH}_3)_2\text{NCH}_2\text{COOCH}_3$, are interconvertible at higher temperatures; between 135° and 293° betaine is the more stable, above 300° a 50% yield of the ester is obtained in the distillate.

Betaine has also been obtained by the oxidation of choline, by the action of chloracetic acid on trimethylamine, and by treating a potassium hydroxide solution of glycocoll with methyl iodide and methyl alcohol.

Betaine crystallises from alcohol in large deliquescent crystals containing $1\text{H}_2\text{O}$ which is lost at 100° or by exposure over strong sulphuric acid. It is precipitated in scales on adding ether to its alcoholic solution. Betaine is optically inactive, has a sweet taste, is not poisonous, and is neutral to litmus. When heated above 300° , it decomposes, with evolution of trimethylamine, and gives an odour of burnt sugar.

Betaine is not affected by chromic or hydriodic acid; but on boiling or fusion with alkali hydroxide it yields trimethylamine.

The salts of betaine are regarded as direct compounds of $\text{C}_5\text{H}_{11}\text{O}_2\text{N}$ with acids. Betaine hydrochloride, $\text{C}_5\text{H}_{11}\text{O}_2\text{NHCl}$, forms large, stable, monoclinic tables, melting with intumescence at 228° and very readily soluble in water, but nearly insoluble in absolute alcohol (distinction from choline). $(\text{C}_5\text{H}_{12}\text{O}_2\text{N})_2\text{PtCl}_6 \cdot 2\text{H}_2\text{O}$, m. p. 242° , forms large, yellow, efflorescent crystals, deposited from water in hexagonal plates, and from dilute alcohol in hydrated octahedra.

The picrate forms yellow needles, m. p. $180-181^\circ$, and can be used to separate the base in mixtures. The aurichloride is dimorphous; from hot water, on slow cooling, it separates as dull-yellow, star-shaped aggregates crystallising in the regular system and melting at 209° ; crystallised in the presence of hydrochloric acid, bright yellow leaflets, prisms and plates with one truncated angle, of the rhombic system, and melting at $248-250^\circ$, are obtained.

Betaine derived from the plant world will pass through the animal body practically unchanged, but it has never otherwise been found in the animal body, nor is there evidence that it is ever originated there, either from choline or otherwise.

Neurine. Vinyl-trimethylammonium hydroxide



This base, discovered by A. W. Hofmann, differs from choline (page 321) by the elements of water. It is believed that neurine occurs in nature only as the result of certain bacterial decompositions of choline. Neurine is not formed in the ordinary hydrolysis of the white matter of the brain by barium hydroxide solution.

Free neurine hydroxide, $C_6H_{13}ON \cdot 3H_2O$, may be obtained by shaking $BrN(CH_3)_3 \cdot CH_2CH_2Br$ with freshly precipitated silver oxide in concentrated solution, filtering and evaporating in a high vacuum at $5-10^\circ$ over frequently renewed P_2O_5 . It forms exceedingly hygroscopic crystals which attack the skin, and decomposes in a few hours at ordinary temperatures. In solution it has a strong alkaline reaction, and absorbs carbon dioxide from the air.

Choline can be converted into neurine by heating it to 140° with fuming hydriodic acid, and eliminating the iodine from the product by moist silver oxide. The reverse reaction has also been effected by heating neurine chloride with hydriodic acid, and then heating the product with silver nitrate in aqueous solution.

Neurine and choline present very close resemblances, and hence the few distinctions between them are important. Thus neurine hydrochloride gives an abundant precipitate with tannin, whilst the choline salt is not affected. On the other hand, choline hydrochloride is precipitated by phosphotungstic acid, which with neurine gives no reaction. Choline platinichloride forms large, soluble, red, tabular, monoclinic crystals, arranged like steps. The platinum salt of neurine, $(C_6H_{12}NCl)_2 \cdot PtCl_4$, crystallises in small, individual, orange-red, regular octahedra, which dissolve with difficulty in hot water. The crystals soon become opaque, and, on treatment with water, leave an insoluble residue, whilst the platinum salt of choline is found in the solution. Neurine and choline may be separated from each other most readily by fractional crystallisation of these salts.

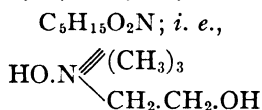
Neurine picrate forms long feathery golden yellow needles, m. p. $263-264^\circ$, more soluble in hot water than in cold, readily soluble in hot alcohol. The aurichloride, m. p. $232-238^\circ$, forms large golden yellow acicular crystals, not very soluble in water.

F. Marino-Zuco (*Gazzetta*, 13, 431) has pointed out that neurine hydrochloride is not decomposed by sodium hydrogen carbonate, in which it differs from the hydrochlorides of most of the poisonous vegetable alkaloids. Hence if the mixed alkaloids and putrefaction

bases simultaneously extracted by Stas's process, are dissolved in hydrochloric acid, and the solution treated with sodium hydrogen carbonate, the vegetable alkaloids can be extracted by agitation with ether, chloroform, amyl alcohol, etc., while the neurine and soluble putrefaction bases remain in the aqueous liquid.

Neurine is extremely poisonous, the symptoms produced resembling those due to poisoning by muscarine. Administered to a frog subcutaneously, it soon produces paralysis of the extremities, which is followed by stoppage of the respiration, and finally of the heart (in diastole). In rabbits, neurine occasions profuse nasal secretion, salivation, and paralysis. Neurine produces contraction of the pupil, both when injected and when applied locally. Atropine has been found to be an efficient antidote, and even to produce temporary immunity to poisoning by neurine. Hydroxy-trimethyl-ammonium compounds are stated by V. Cervello to act similarly.

Choline. Trimethylhydroxyethylammonium hydroxide.



Choline has been prepared synthetically by a number of methods. The most convenient appear to consist in treating trimethyl-amine with dry ethylene-dibromide at $110\text{--}112^\circ$ to form trimethylamino-bromethylum bromide, and to heat this with 25 parts of water at 160° for a few hours, or to pass rather more than one equivalent of trimethylamine gas into ethylene chlorhydrin cooled to -12° to -20° in a tube which is subsequently warmed to $80\text{--}90^\circ$. The yield by the latter method is almost quantitative.

Choline is a decomposition product of some lecithins, but also exists ready-formed in the tissues of living animals and plants, and is one of the first and most constant products of the putrefactive decomposition of nervous tissue. It was first isolated from bile (whence the names choline and bilineurine), has been found in herring brine, and exists also in the brain and in yolk of egg, in the conjugate form of a lecithin.

It is the constant associate of neuridine during the earlier stages of putrefaction, being afterwards replaced by trimethylamine,

which is, no doubt, produced by the decomposition of choline itself— $C_5H_{15}NO = C_2H_6O_2 + C_3H_9N$.

Besides occurring naturally in the animal kingdom, choline exists in a large number of plants and plant products.

Choline may be prepared from yolk of egg or lecithin by exhausting the substance with ether and afterwards with warm alcohol. These are distilled from the extract, the residue boiled for an hour with barium hydroxide, the excess of barium precipitated by carbon dioxide, and the filtrate evaporated. The residue is exhausted with absolute alcohol, and the solution precipitated with platinic chloride. The platinichloride is dissolved in water, and decomposed with hydrogen sulphide. The solution of choline chloride is concentrated, and treated with silver oxide, when a strongly alkaline solution of free choline is obtained, and on evaporation the base remains as a syrupy liquid.

Free choline is a deliquescent substance. It usually forms a syrup, but can be obtained in crystalline form by leaving it in a vacuum desiccator over P_2O_5 . It is a powerful base, having an alkaline reaction, and absorbing carbon dioxide from the air. It is very soluble in water, from which it cannot be extracted by organic solvents.

Choline, having the constitution of a tetralkylammonium hydroxide (compare Vol. VII), forms salts by the replacement of the OH group by Cl, I, SO_4 , etc. Thus the *chloride* has the formula $(C_2H_4OH)(CH_3)_3N.Cl$, and crystallises from absolute alcohol in fine deliquescent needles, readily soluble in alcohol and water (distinction from betaine). The platinum salt has the composition $[(C_2H_4OH)(CH_3)_3NCl]_2.PtCl_4$, and crystallises from hot water in fine reddish-yellow plates or prisms, insoluble in absolute alcohol. It is dimorphous. The gold salt is deposited from a hot saturated aqueous solution in long yellow prisms, which melt at 244 to 245° , and are soluble with difficulty in cold water, but dissolved by hot water or hot alcohol. Choline sulphate is amorphous, and almost insoluble in absolute alcohol, but very soluble in water.

The most sensitive precipitant is potassium triiodide. A concentrated solution it is said to precipitate 1 part in 2,000,000. Choline periodide forms rhombohedral, almost quadratic, leaflets. This test has been used as a quantitative method.

When choline is treated with hydriodic acid, both the hydroxyl groups are replaced, with formation of the substance $(C_2H_4I)(CH_3)_3NI$; and when this is treated with moist oxide of silver and water (compare Vol. VII), it yields the base neurine, $(C_2H_3)(CH_3)_3N.OH$. On oxidation choline is converted into betaine, $(CH_2.COOH)(CH_3)_3N.OH$.

Choline exists pre-formed in the tissue of the adrenal body, and indeed in many other organs. It is present in the blood in degenerative diseases of the central nervous system. It is commonly regarded as antagonistic to epinephrine, but this is very doubtful. The presence of choline in the body is to be regarded as the result of the normal disintegration of nervous tissue, and the bile is probably the fluid of elimination. Choline has been reported in the urine in Addison's disease.

Schulze's method for separating choline from a mixture of bases is as follows:¹—An aqueous extract of the material (which is preferable to an alcoholic one since it excludes phosphatides more completely) is purified with lead acetate, strongly acidified with sulphuric acid and precipitated with phosphotungstic acid. After regeneration of the precipitate with baryta, the purine bases and the histidine and arginine fractions are removed by means of silver nitrate in the usual manner, and the last filtrate is again precipitated with phosphotungstic acid; after regeneration the mixture of chlorides is dissolved in 95% alcohol and precipitated with alcoholic mercuric chloride. Choline mercurichloride is very little soluble in boiling water, the betaine compound more so. The separation is completed by converting the more and the less soluble mercurichlorides into the dry hydrochlorides and extracting them with anhydrous alcohol, which leaves betaine hydrochloride undissolved. The method may be shortened by omitting the second precipitation with phosphotungstic acid, and in place of it precipitating the filtrate from arginine at once with mercuric chloride (after removal of the silver). It is also possible to combine Staněk's process (cf. page 324) with mercuric chloride precipitation.

The properties of trigonelline are similar to those of betaine, and the separation from choline is effected in the same way. According to Schulz 3-4% of these bases escape precipitation with phosphotungstic acid. In alcoholic solution 5 per cent of the

¹ From G. Barger, "*The Simpler Natural Bases*," 1914, pp. 150-152.

trigonelline and choline escaped precipitation by mercuric chloride, but in the case of betaine the loss was more than double this amount, so that it is advisable to concentrate the filtrate.

The method of Staněk for separating and estimating choline and betaine is based on the fact that betaine is set free from its salts by sodium bicarbonate, whilst choline is not. To 25-40 c.c. of the aqueous solution containing at most 5 per cent of the two hydrochlorides, sodium or potassium bicarbonate is added to make 5 per cent and then a solution of 153 grms. of iodine and 100 grms. of potassium iodide in 200 grms. of water is added until precipitation is complete; the precipitate consists of brown choline periodide and soon becomes crystalline. It is collected on a paper disk in a Gooch crucible, washed with water and transferred to a Kjeldahl flask for nitrogen determination. If desired, the choline may, instead, be recovered from the periodide by adding finely divided ("molecular") copper, boiling with cupric chloride and copper, and, after filtration, treating the filtrate with hydrogen sulphide. The solution contains the choline as hydrochloride.

The betaine is estimated in the filtrate from the choline periodide by concentrating, acidifying with sulphuric acid, saturating with sodium chloride and precipitating as the periodide with the potassium triiodide solution. A nitrogen determination gives the betaine content.

The following method for estimating choline in blood has been devised by J. S. Sharpe (*Biochem. J.*, 1923, **17**, 41). From 20 to 50 c.c. of blood are drawn off into about 4 times this volume of absolute alcohol and well shaken. The containing vessel is allowed to stand for about 12 hours, after which time the contents are filtered into an evaporating basin. The precipitate is rubbed up and well washed with absolute alcohol and then added to the filtrate. This is evaporated to a syrup and taken up in distilled water. The possibility of the extraction and decomposition of lecithin, with the liberation of choline, at this stage is considered later. The fluid is again evaporated to a syrup, and the process repeated until it is alcohol-free. The solution is then transferred to a dialysing thimble of about 15 c.c. capacity and dialysed for 24 hours into 200 c.c. of distilled water. It is advisable to repeat this dialysis and to combine the resulting liquids. The mixture is evaporated to about 5 c.c. and carefully washed into a beaker, the volume being kept

as low as possible. 20 to 30 volumes of a saturated solution of iodine in potassium iodide are now added, and the beaker set aside overnight to allow the choline periodide to crystallise. The crystals are filtered off on a Gooch crucible containing a layer of fine asbestos wool. They are washed repeatedly with ice-cold water until free from the precipitating solution. The Buchner flask is rinsed with cold water. The crucible is placed in position for filtering, and the choline periodide is now ready for decomposition. This is accomplished by means of a mixture of 2 parts of dilute nitric acid (1 part HNO_3 to 1 part water) with 1 part chloroform. The above mixture is then shaken and added to the crucible. The periodide is decomposed by the nitric acid, and the free iodine is taken up by the chloroform and carried into the flask by the suction. This is repeated several times until the asbestos is quite white. The crucible is now removed, more chloroform is added to the flask and well shaken in order to extract any iodine dissolved in the water. The chloroform layer is then washed free from acid by decantation with repeated quantities of cold water. The liberated iodine in the chloroform layer is now most conveniently titrated with $N/20$ sodium thiosulphate solution until the pink colour just disappears. The possibility of lecithin breaking down in this procedure is small if the process is carried out with reasonable speed. Tests made on blood to which no choline was added gave negative results. If there was any free choline present, its concentration was below the limit of recovery by this process.

In addition to the bases or nitrogenous bodies which have been or will be described here, a number exist which have marked physiological actions. Some have not been isolated in a state of chemical purity; others, while isolated in this way, have not had their chemical structures definitely determined. It will not be possible or advisable to enter into the description of all of these substances in the present connection. Reference must be made to special compilations¹ and periodic reviews for their enumeration, especially since it is impossible to predict which ones may become of special importance and significance in scientific and practical studies at any moment; one more substance of this group will, however, be mentioned here. **Muscarine**, the poisonous principle of the toadstool known as the fly agaric (*Agaricus muscarius*), present in the fungus *Amanita*

¹ Such as "The Simpler Natural Bases," by G. Barger.

pantherina, and formed also in the putrefaction of fish, was described in the former editions of this work, and has been taken to possess the chemical structure of choline aldehyde. The work of H. King (*J. Chem. Soc.*, 1922, **121**, 1743) has thrown a different light on the relations involved. He has shown that the formula assumed heretofore for the substance is incorrect. His method of isolation of muscarine from *Amanita muscaria* is as follows:—The isolation of muscarine depends on its solubility in absolute alcohol, and on its immunity from precipitation by basic lead acetate or colloidal iron. It was only partially precipitated together with choline, by aqueous mercuric chloride in neutral solution. A much larger proportion was precipitated by alcoholic mercuric chloride, and a further quantity by repeating the process after intermediate removal of some inhibiting substances. Finally, the residual muscarine was completely precipitated by phosphotungstic acid. Muscarine can also be precipitated completely, in the absence of inhibiting substances, by saturating its alcoholic solution with mercuric chloride.

The distribution of active material is shown by the following table:

Aqueous HgCl ₂ ppt.	First alcoholic HgCl ₂ ppt.	Second alcoholic HgCl ₂ ppt.
14%	34%	15%
Phosphotungstic acid ppt.		Total recovered
17%		80%

The combined precipitated bases thus obtained consisted chiefly of choline and muscarine in a ratio of the order of 20:1, accompanied by one or more substances, of which one, at least, is laevorotatory. The major part of the choline was separated by fractionation as the *d*-hydrogen tartrate from 95% alcohol by Honda's method, which is difficult, but efficient. Fractionation was then continued by means of the aurichlorides, choline aurichloride being again the less soluble. From the mother liquors, muscarine aurichloride was finally isolated in large delicate leaflets totally distinct from choline aurichloride and from Harnack's description of muscarine aurichloride, but not inconsistent with Nothnagel's description. On analysis it was found to contain 38% of gold, whereas Harnack and Nothnagel found 43.0 and 42.9%, respectively.

25.5 kg. of fresh material gave only 0.4 grm. of muscarine chloride. The physiological activity of the isolated product was much greater

than any previously reported, 0.002 mg. stopping the frog heart in diastole. The physiological action on an isolated loop of rabbit's intestine was also studied.

The molecular or equivalent weight of muscarine chloride was found to be 210 (choline chloride 137). It had a greater complexity than choline. No evidence for its containing one atom of oxygen more than choline was obtained, and there was no satisfactory evidence that it behaved as a quarternary base. Muscarine seems to rank with the alkaloidal bases of greater complexity, rather than with choline. The activity was unchanged after boiling with one-tenth normal acid or alkali.

PHOSPHATIDES (LECITHINS)

Certain esters of phosphoric acid which resemble fats, and which are extracted from animal and plant tissues by the usual fat solvents, are termed phosphatides. A number of preparations have been described at various times as definite substances depending upon certain arbitrary standards, such as the ratio of nitrogen to phosphorus equivalents, or certain physical properties. The recent careful studies of these substances, especially by P. A. Levene and his co-workers, resulted for the first time in the preparation of an analytically pure lecithin, and showed that only three generic substances, lecithin, cephalin, and sphingomyelin,¹ should, at the present time, be included in the group of phosphatides whose chemical structures are definite.

Phosphatides are found in practically all animal tissues, especially in brain, nerve tissue, eggs, etc., and in most vegetable tissues. Hot alcohol extracts all the phosphatides, but the other fat solvents extract only certain of them. If pure lecithin or cephalin is desired, acetone is used. Ether is used for the preparation of all unsaturated phosphatides. The dried tissues are extracted directly for sphingomyelin. In carrying out the extractions, all three reagents are employed consecutively on the organ minced and dried in a vacuum drier. The three extracts contain the following substances:—

The acetone extract contains lecithin and cephalin and only a small proportion of their decomposition products.

¹ Cuorin, which was included by MacLean in his Monograph on "Lecithin and Allied Substances," was shown by Levene and Komatsu to be a mixture of the decomposition products of lecithin and cephalin.

The ethereal extract contains lecithin and cephalin, together with products of their partial decomposition. This fraction can be separated into two parts: one, soluble in cold and in hot alcohol and commonly named "lecithin," and one, soluble in ether but insoluble in alcohol, commonly named "cephalin."

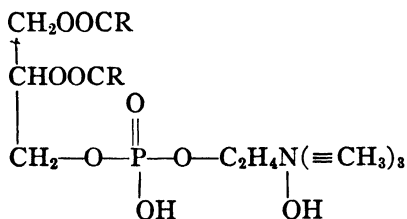
The alcohol extract of dried brain, on standing, forms a white precipitate, the "White Matter" of Vauquelin.

For the purification of the individual phosphatides various reagents have been used. Cadmium chloride forms a useful insoluble double salt with lecithin which, after separation, may be decomposed again to form the lecithin by hydrogen sulphide, ammonium carbonate, or perhaps best by methyl alcohol saturated with gaseous ammonia.

Cephalin may be separated from lecithin by means of the cadmium salts, that of the former being soluble in ether, that of the latter insoluble, and also by means of alcohol, in which the cephalin, when pure, is insoluble.

Sphingomyelin may be prepared from the "White Matter" of Vauquelin (also known as Protagon) by fractional precipitation from alcohol and by purification by means of cadmium chloride. Fractional precipitation from pyridine has also been of value here.

The following structure of lecithin is now generally accepted:—



It is a compound of phosphoric acid with a diglyceride and choline. (In some of the older work on lecithin somewhat different conclusions were drawn, because of the presence of cephalin as impurity.) The groupings indicated by R and R_1 form parts of the fatty acids combined with the glycerol residue. It is only since Levene was able to obtain lecithin in a pure state that it has been possible to identify these acids and fix the structure of lecithin with certainty. It was found that the lecithin from egg yolk and the liver of cattle contained the same fatty acids, namely, one unsaturated and two

saturated acids, the former being oleic acid and the latter palmitic and stearic acids. Liver lecithin appeared to contain a different unsaturated acid, but the same saturated acids. The former belonged to the linolic series, or possibly two acids, one belonging to the oleic series and the other to the linolic series, were present. The lecithin structure, as given, admits of only two acid groups. Further study showed that in each case (egg, brain, or liver) probably several lecithins were present, each containing two of the acid groups in a molecule, the substance isolated consisting of a mixture each time. The degree of unsaturation of the lecithins, that is, their iodine values, serves to establish the ratios of the amounts of unsaturated to saturated acids present and should prove of value in characterising the pure substances. In the heart (W. R. Bloor), the phospholipid fatty acids consist of 10–20% of saturated, 50–70% of unsaturated, and about 25% of acids of unknown nature. Of the unsaturated acids, the lecithin of ox heart contained 45% of linolic, less than 6% of arachidonic, and the rest oleic. Catalytic hydrogenation of lecithin in alcoholic solution in the presence of colloidal palladium results in the formation of dihydrolecithins which can be handled more readily in the laboratory and studied more advantageously than the natural substances. The action of cobra venom on lecithins, or on the egg yolk, etc., containing the lecithins, hydrolyses the lecithins, in the sense that the unsaturated acid residues are split off, leaving lysolecithins. These last compounds can readily be handled and are serving as the basis for the preparation of synthetic lecithins by the introduction of different acyl groups. Further work along these lines will probably clear up many of the unknown relations among these compounds.

The following method for preparing pure amino-free lecithin from egg yolk, brain, and liver tissue within 24 to 48 hours has been described by P. A. Levene and I. P. Rolf (*J. Biol. Chem.*, 1927, **72**, 587).

Preparation of Crude Cadmium Chloride Salt of the Lecithin Fraction

Egg Yolk.—A homogeneous emulsion (by means of mechanical stirrer or egg beater) of fresh egg yolks is strained through cheese cloth and poured into twice its volume of hot 95% alcohol. After the alcoholic extract has cooled, a cold saturated cadmium chloride

solution in methyl alcohol is added to produce complete precipitation of the lecithin.

Liver Tissue.—40 lbs. of fresh liver are freed from adhering adipose tissue, ground, dried, and extracted with 28 litres of 95% alcohol. The alcoholic extract is concentrated to one-third of its volume, kept overnight at 0° to allow the white matter to crystallise, and to the filtrate is added enough of a cold methyl alcohol solution of cadmium chloride to precipitate all the lecithin.

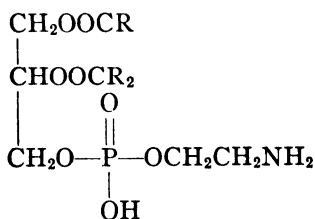
Brain Tissue.—40 lbs. of brain tissue, freed from adhering foreign matter, are minced, dried in a vacuum drying oven, extracted twice with acetone (16 litres) and then with 24 litres of hot 95% alcohol. The alcoholic extract is then treated as with liver tissue.

Purification of Cadmium Chloride Salts and of Lecithins.—To avoid decomposition of the lecithins in the course of their preparation, high temperatures must be avoided, and the use of water reduced to a minimum. The principal impurity present is cephalin. To remove this, the cadmium salts are well shaken with ether and the suspension centrifuged. This is repeated 8 to 10 times. The salts are then suspended in chloroform (400 c.c. per 100 grm.), the suspension shaken until a slightly opalescent solution is formed, and a cold 25% solution of ammonia gas in dry methyl alcohol added as long as a precipitate forms. The precipitate is removed by centrifuging, extracted with chloroform, and the solution treated with methyl alcoholic ammonia. The combined chloroform and methyl alcohol solutions of the lecithins are concentrated at 10–15 mm. pressure, on the water-bath at 35–40°. The residue is dissolved in dry ether and evaporated to dryness as before. This is repeated 3 times. The final residue is extracted with 99% alcohol, the cephalins remaining undissolved. The solution is treated with a methyl alcoholic cadmium chloride solution and the lecithins liberated as before. The cadmium-free dry residue is dissolved in a minimum volume of ether and poured into acetone (500 c.c. for the residue from 100 grm. of cadmium salts). The final purification (removal of small quantities of ammonia) is accomplished by dissolving 50 grm. of lecithin in 50 c.c. of ether, adding an equal volume of 10% acetic acid, shaking on a shaking machine for $\frac{1}{2}$ to 1 hour, pouring the thick emulsion formed into 500 c.c. of acetone, decanting the supernatant liquid, and washing the precipitate repeatedly with dry acetone. The wash acetone is added to the decanted water-

acetone solution, and the combined solution evaporated under reduced pressure to dryness. The residue is dissolved in dry ether, and the lecithin precipitated with acetone. The first precipitate, the purer material, contains about 50% of the original lecithin, the second about 25%.

Cephalin may be prepared as follows (Levene and Rolf, *J. Biol. Chem.*, 1927, **74**, 713):—"40 lbs. of brain tissue, freed from membranes, are minced in a hashing machine and then dried in a vacuum drier. The dry material is then pulverised, and the drying is continued. The product is exhaustively extracted with acetone. This extraction requires about 20 litres of acetone. The residue is freed from acetone in the vacuum drier and thoroughly extracted with 95% alcohol. (30 litres of alcohol are required.) The residue after this extraction is extracted with 20 litres of ether, and the ethereal extract concentrated to small volume and allowed to stand at 0° overnight to allow the contaminating "white matter" to settle out. The latter is removed by centrifugalisation, and the supernatant liquid poured into 98.5% alcohol warmed to 60°. The precipitate thus formed is dissolved in ether, and the ethereal solution is again allowed to stand at 0° overnight. The operations of precipitation with alcohol and redissolving in ether are continued as long as the ethereal extract, on standing, deposits a sediment of white matter. The product as finally formed by precipitation from alcohol, contains no non-amino nitrogen. The yield is 18.0 gm.

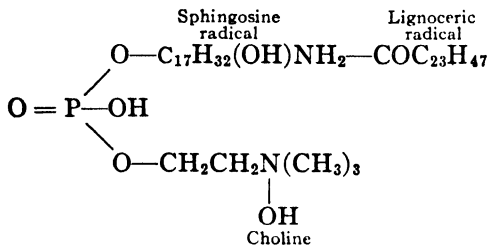
The structural formula for cephalin may be written as follows:



The essential difference from lecithin is the basic group, amino-ethanol in place of choline. Although the question of the fatty acids present is not finally settled, the evidence at present favours the view that one unsaturated (linolic) acid and one saturated (stearic) acid are present. The unsaturated acids of cephalin from ox heart consist of 6% arachidonic and the rest mainly linolic

(Bloor). Lysocephalin has been prepared and studied. Although considerable evidence has been collected indicating the presence of other cephalins, further work will be required to clear up the nature of these bodies and to decide whether chemical individuals or mixtures are present.

Sphingomyelin has been assigned the following structure:



It is evidently a diamido-monophosphatide, as compared with lecithin and cephalin, which are monamido-mono-phosphatides.

Sphingomyelin is a compound of phosphoric acid, an organic acid, and the two bases sphingosine and choline. Phosphoric acid may be liberated by the action of either acid or alkali, choline by hydrolysis with barium hydroxide. Sphingosine probably has the structure $\text{CH}_3(\text{CH}_2)_{11}\text{CH}:\text{CH}.\text{CH}(\text{OH}).\text{CH}(\text{OH}).\text{CH}_2\text{NH}_2$, an unsaturated dihydroxy-monamino compound.

The formula of sphingomyelin, as given, shows a fatty acid radical. Recent work indicates the occurrence of two fatty acids, so that either the simple molecule as indicated must be doubled to permit of their inclusion, or the existence of different sphingomyelins must be assumed with the simple molecule, the difference lying in the character of the fatty acids. This is the same question which was involved in the structure of lecithin, where it was decided in favour of different lecithins of the simple structures.

The phosphatides, in general, are translucent and of soft wax-like consistence. Their solubilities have been given in connection with their separations from each other. In practical working, it is best to dry the tissue in question first, preferably by heating *in vacuo*, although air drying or washing with acetone have also been recommended, before grinding to a powder and proceeding to the extractions.

Their chemical properties are fairly evident from a consideration of their structures. They are amphoteric and unsaturated in

character. Cephalin gives off its nitrogen in the Van Slyke amino nitrogen method. Since the different substances have been isolated in a pure state comparatively recently, their chemical reactions have not been studied very extensively as yet. Their physiological properties are important, but here also the fact that mixtures have generally been used, makes the description of the individual substances of less significance. For example, the view that lecithin could function as a vitamin has been shown to be due to the fact that the experiments on which it was based involved the use of impure lecithin. When the lecithin was purified it lost its vitamin activity. The questions of fat metabolism and of blood coagulation involve, however, the phosphatides, and now that the pure substances are available, these relations can be studied more satisfactorily.

This account of the phosphatides (or lecithins) does not include the general methods of preparation based upon the older work. The recent studies have shown that such methods are not useful for the scientific study of the individual substances. For those who wish to use the older methods in connection with certain practical problems, reference may be made to the former editions of this work. The more recent work in this field can best be obtained from H. MacLean's monograph on "Lecithin and Allied Substances" to which reference has already been made, and to a review¹ entitled "Structure and Significance of the Phosphatides" by P. A. Levene, with a bibliography by I. P. Rolf.

CEREBROSIDES

Several basic substances, similar in solubility relationships to the fats and the phosphatides but containing no phosphorus, have been isolated from brain. They have been called cerebroside. Two of these, phrenosin and kersin, will be described.²

The cerebroside are substances of glucoside nature which on hydrolysis decompose into a reducing sugar (galactose), a base (sphingosine) and a fatty acid. The fatty acid of phrenosine is phrenosinic acid, $C_{23}H_{47}CHOH.COOH$ (the α -hydroxy derivative of the next higher homologue of lignoceric acid); that of kersin is lignoceric acid, $C_{24}H_{48}O_2$.

¹ *Physiological Reviews*, 1921, **1**, 327.

² Cf. especially H. MacLean "The Lecithins and Allied Substances."

They possess many of the properties of sphingomyelin. The latter is rather more soluble in cold absolute alcohol and less soluble in cold pyridine than the cerebrosides, a property which is taken advantage of in their separation. When dry the cerebrosides are white and more or less opaque, but are capable of becoming, in part, transparent like wax. They dissolve easily in hot alcohol, from which they are deposited on cooling. In cold absolute alcohol and in cold benzene they are practically insoluble; in hot benzene they dissolve easily, and are deposited, on cooling, as a gelatinous mass. The addition of cold alcohol to a hot benzene solution precipitates the cerebrosides in the form of white flakes. They are almost insoluble in cold or hot ether. They dissolve readily in pyridine at 30° , but are insoluble in cold acetone. From hot alcoholic or hot acetone solutions, the phrenosin constituent is first precipitated on cooling, and only at lower temperatures after most of it has separated, does the kerasin precipitate. This property is used in the separation of the two cerebrosides. True crystals of phrenosin can be obtained, but up to the present not of kerasin, although both form "liquid crystals" at certain temperatures.

Phrenosin is dextrorotatory and kerasin laevorotatory when dissolved in pyridine.

A number of methods have been described for the isolation of the cerebrosides.

Perhaps the most satisfactory method is that of Rosenheim. According to this method, water and cholesterol are extracted from brain by repeated treatment with acetone, the unsaturated phosphatides with cold petroleum spirit, and the cerebrosides dissolved out by pyridine. They are precipitated by pouring the solution into 3 to 4 volumes of acetone and cooled to 0° . After being washed with acetone and extracted in a Soxhlet apparatus with ether to remove the last traces of the phosphatides, they are recrystallised several times from an alcohol and chloroform mixture (1:2) and are obtained in pure white condition.

Phrenosin and kerasin may be separated from each other by means of their different solubilities in acetone containing 10% water, the former separating at 37° , the latter at 0° . The former is then purified by recrystallising from a chloroform and glacial acetic acid mixture (2:3), and finally from aqueous (10%) acetone, and the latter by precipitation first from the chloroform and acetic acid

mixture, then from acetone and pyridine mixture (1:1), and finally from acetone containing a little pyridine.

By a long series of extractions with ethyl alcohol, petroleum spirit, chloroform with methyl alcohol, and precipitation by methyl acetate, a small quantity of pure kersin was obtained from 45 kilos. of fresh spleen. It was identified by its cleavage products, lignoceric acid, sphingosine and galactose.

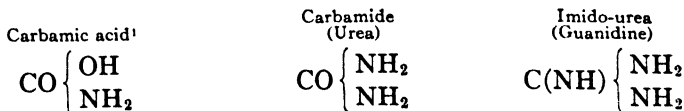
A new cerebroside called *Nervon* was isolated from both beef and human brain (E. Klenk). It was found in the ethereal or petroleum spirit fraction, together with the unsaturated phosphatides, and is very similar in its properties to kersin. Its cleavage products are galactose, sphingosine, and a new unsaturated fatty acid, $C_{24}H_{46}O_2$, m. p. 41° . The formula of nervon is given as $C_{47}H_{89}O_8N$.

Brain and nerve tissue form the principal sources for the cerebrosides, although they appear to occur in most tissues. Probably the compositions are different when obtained from different sources, although direct evidence is lacking.

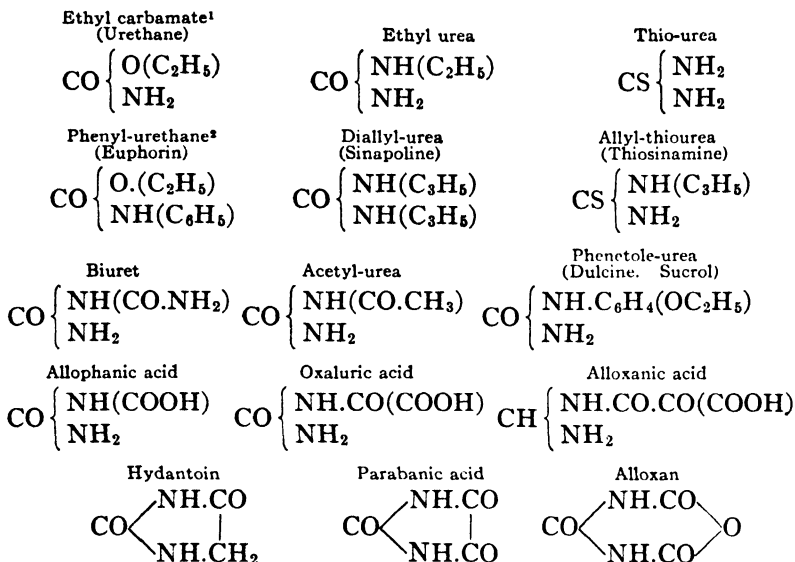
Quantitative estimation of the cerebrosides present in a tissue is difficult, although some methods have been suggested. The method of separation developed by Rosenheim may be used. A determination, using the crude product obtained in the separation, gave a content of cerebrosides of 2% for wet brain and 9% for dry brain.

UREA AND ITS ANALOGUES

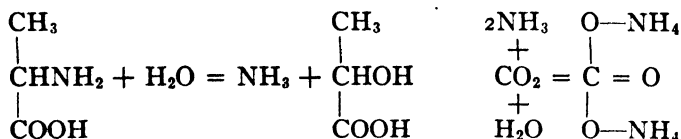
Urea is itself of pre-eminent interest and importance as the chief form in which the nitrogen of the protein ingested by man and other mammalia is eliminated from the system. Urea is also the type of an extensive series of allied bodies and the nucleus of other compounds of natural origin and artificial synthetic formation. The following is a list of the simpler and more typical members of the group:—



¹ Carbamic acid, NH_2COOH , is not known in the free state. The ammonium salt is formed by the direct union of carbon dioxide and dry ammonia gases, a second molecule of ammonia uniting then with the acid to form ammonium carbamate, $NH_2COO(NH_4)$. This salt exists in commercial ammonium carbonate, and can be obtained by digesting that



The larger portion of urea formed in the animal body is probably derived as follows. The amino-acids, products of the hydrolysis of protein, are deaminised, and the ammonia combined with the carbon dioxide of the circulating fluids to form ammonium carbonate. This is then converted into urea, through ammonium carbamate as intermediary stage. Thus for alanine:



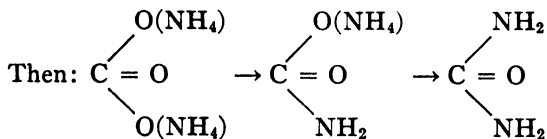
compound in strong ammonia for 30 to 40 hours (Divers). Ammonium carbamate is extremely soluble in water, with which it gradually reacts to form ammonium carbonate ($\text{NH}_4\text{O.CO.O}(\text{NH}_4)$). When heated to about 60° , at the ordinary pressure, ammonium carbamate is decomposed into ammonia and carbon dioxide, but when heated under pressure to $130-140^\circ$ it yields urea.

Calcium carbamate is precipitated on adding lime and alcohol to a solution of ammonium carbamate cooled to 0° . It forms a crystalline powder, soluble in water. The solution rapidly decomposes, with separation of calcium carbonate.

Salts of carbamic acid occur in serum, and are also stated to be formed by the oxidation of leucine, tyrosine, glycocholic, and albumin by potassium permanganate in alkaline solution.

¹ Ethyl carbamate or Urethane, $\text{NH}_2.\text{CO.O}(\text{C}_2\text{H}_5)$, results from the action of aqueous ammonia on ethyl carbonate. It is also formed by the action of alcohol at 100° on urea or urea nitrate, and may be obtained by other methods. Traces of urethane exist in urine. Ethyl carbamate melts at about 50° , and distills at 182° . It is sparingly soluble in water, but readily soluble in alcohol and in ether. Treated in the cold with alcoholic potash, it yields crystals of potassium cyanate, KCNO . When heated with ammonia, urethane is converted into urea.

² Phenyl-urethane, $\text{NH}(\text{C}_6\text{H}_5).\text{CO.O}(\text{C}_6\text{H}_5)$, has been employed medicinally, as an antipyretic and antirheumatic under the name of "euphorin." Acetyl and propionyl derivatives of hydroxyphenyl-urethane, have been prepared and proposed as remedies by E. Merck,

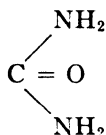


one molecule of water being extruded in each stage.

Guanidine is described on page 350, and biuret on page 339. Thiosinamine is described under mustard oil *q.v.* while oxaluric acid, parabanic acid, and alloxan are referred to under uric acid. Urea and dulcine are the only remaining members of the group which require further consideration.

Urea. Carbamide

CH_4ON_2 ; *i. e.*,



Urea exists in the urine of mammals, and in blood, milk, and other animal fluids. In the circulating fluids and tissues, urea exists in traces; there is normally no threshold value for urea in the kidney; the blood is practically freed from urea by passing through the kidney. The urea is formed largely in the liver, not at all in the kidney apparently. It is also eliminated to some extent in the stool and perspiration, and with free natural or forced sweating relatively large amounts of urea may be thus removed. It was first obtained synthetically by Wöhler in 1828, being the first of the natural organic bodies prepared by a synthetic process.

Urea may be prepared by a variety of methods, of which the following are the most important and interesting:

1. Fresh urine is concentrated at 100° to one-tenth of its volume, and the insoluble deposit of phosphates and urates separated by filtration. The filtrate is mixed with an equal volume of a hot concentrated solution of oxalic acid, and the whole vigorously agitated and allowed to cool. A copious, fawn-coloured precipitate of oxalate of urea is obtained, which is separated by a filter, slightly washed with cold water and pressed. The product is dissolved

in boiling water, and powdered chalk added till the liquid becomes neutral and effervescence ceases. The liquid is filtered from the calcium oxalate, warmed with animal charcoal, filtered, and concentrated by evaporation, actual boiling being avoided. The urea which deposits on cooling is purified by recrystallisation from alcohol.

2. Liebig and Wöhler's classical method of preparing urea affords an interesting example of rearrangement of the atoms in the molecule. Both ammonium cyanate and urea have an elementary

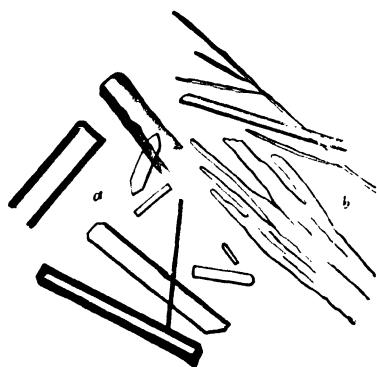


FIG. 16.—Urea; *a*, quadrilateral prisms; *b*, indefinite crystals, as deposited from alcoholic solutions.

composition corresponding to the empirical formula: CH_4ON_2 . On evaporating an aqueous solution of ammonium cyanate at the temperature of boiling water, the salt suffers molecular change into urea, according to the equation: $\text{CN.O}(\text{NH}_4) = \text{CO}(\text{NH}_2)_2$. The conversion is never quite complete.

In carrying out Liebig's reaction in practice, it is not necessary to operate on pure ammonium cyanate. Potassium cyanate in strong aqueous solution is treated with an equal weight of ammonium

sulphate, and the whole evaporated to dryness on the water-bath. The product is boiled with strong alcohol, which dissolves the urea, leaving a residue of potassium and ammonium sulphates. On concentrating and cooling the alcoholic solution, crystals of urea are deposited. Instead of employing potassium cyanate previously prepared, it may be extemporised by heating a mixture of 28 parts of dehydrated potassium ferrocyanide and 14 parts of manganese dioxide in an iron vessel till it becomes sticky. The product is extracted with cold water, evaporated to dryness with 20.5 parts of ammonium sulphate, and the residue extracted with alcohol as before.

3. Urea has been obtained in small quantities by passing a current of air mixed with ammonia and benzene vapour over heated platinum wire (*J. Chem. Soc.*, 39, 471), and by passing ammonia and carbon dioxide through a red-hot tube.

Urea forms transparent, colourless, four-sided, somewhat hygroscopic anhydrous prisms (Fig. 15). It is odourless and possesses a cooling saline taste, like that of nitre. When heated to 132° urea melts, and at 150 to 160° decomposes with evolution of ammonia and formation of biuret, $C_2H_5O_2N_3$,¹ which on further heating splits into ammonia and ammonium cyanate, leaving a residue containing melanuric acid, $C_3N_3(OH)_2NH_2$, and cyanuric acid, $C_3H_3O_3N_3$, which bears a much stronger heat without change. In a vacuum, urea distils unchanged at 135° .

The solubility of urea in grm. per 100 grm. of water at temperatures between 0° and 70° is as follows (L. A. Pinck and M. A. Kelly, *J. Amer. Chem. Soc.*, 1925, **47**, 2170):—

0°	10°	20°	30°	39.7°	50°	60°	70°
67.0	84.0	104.7	136.0	165.4	205.0	246.0	314.6

Urea is also soluble in five parts of alcohol at ordinary temperatures, and one part at the boiling temperature, and dissolves in amyl alcohol, but it is nearly insoluble in ether, and quite so in chloroform and essential oils.

At the ordinary temperature an aqueous solution of pure urea has practically no tendency to change, but, on boiling, a certain reversion to ammonium cyanate takes place. The transformation ceases in about an hour, when the decomposition is between 4 and 5% (Walker and Hambly, *J. Chem. Soc.*, **67**, 749). When heated with water under pressure urea undergoes hydrolysis, with formation of ammonium carbonate: $\text{CH}_4\text{ON}_2 + 2\text{H}_2\text{O} = (\text{NH}_4)_2\text{CO}_3$.

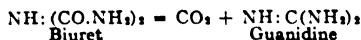
¹ **Biuret**, $\text{NH}:(\text{CO}.\text{NH}_2)_2$, is formed when urea is heated to $150\text{--}160^\circ$, until the fused substance becomes pasty and ceases to evolve ammonia. On treating the product with hot water, cyanuric acid remains undissolved, and biuret crystallises out on concentrating and cooling the filtrate. It may be purified by re-solution in hot water and precipitation with dilute ammonia.

Biuret crystallises from water in long, white acicular crystals, containing $1\text{H}_2\text{O}$ or from alcohol in anhydrous laminae. It is sparingly soluble (1:65) in cold water, but very readily (45:100) in boiling water, and is easily soluble in alcohol. Biuret is dissolved unchanged by cold concentrated sulphuric acid.

Biuret in aqueous solution is not precipitated by tannin, nor by solutions of lead or silver. Its most characteristic reaction is the production of a red or violet solution (the tint varying with the relative proportions of biuret and the reagent employed), on adding sodium hydroxide and a few drops of a solution of cupric sulphate or Fehling's solution. This test, often referred to as "the biuret reaction" affords a means of detecting urea (compare page 342).

Biuret is a weak base, forming salts readily decomposed by water. The cyanurate, $B_3C_3H_3O_3Na_3$, is deposited in needles during the preparation of biuret. It differs from urea cyanurate, for which it has been mistaken, by giving $3NH_3$ instead of $2NH_3$ when boiled with baryta water, and in evolving 14.8 % of nitrogen instead of 11.5 % when treated with alkaline hypobromite.

When heated to a temperature above 170°, biuret is decomposed into ammonia and cyanuric acid. Heated in a current of hydrochloric acid gas, it yields cyanuric acid, urea, and guanidine, together with ammonia and carbon dioxide:



Many bacteria ferment urea, with the production of ammonium carbonate. This occurs in pure solutions of urea, as well as in mixtures containing organic matter. This decomposition often occurs in the bladder, as the result of septic infection, ammoniacal reaction and precipitation of the calcium phosphates and possibly urates occurring in consequence. This bacterial decomposition occurs very easily in urine, since all urine is infected on being voided; therefore, it is necessary to add an antiseptic, such as thymol, chloroform, or toluene, or to refrigerate.

Urea also yields ammonia when fused with alkali hydroxide or ignited with soda-lime, a carbonate being formed at the same time. When heated with a strong mineral acid, urea similarly forms an ammoniacal salt, carbon dioxide being evolved.

When sterile, urea remains unchanged in slightly acid solution; but if the solution contains a fixed alkali, the urea will be slowly converted into ammonium carbonate.

Pure concentrated nitric acid combines with urea without decomposing it, but if the acid contains nitrous acid the urea is resolved into water, nitrogen, and carbon dioxide, according to the following equation: $\text{CH}_4\text{ON}_2 + \text{N}_2\text{O}_3 = 2\text{H}_2\text{O} + 2\text{N}_2 + \text{CO}_2$. With Millon's reagent the reaction occurs promptly and completely, and may be employed for the determination of urea. Chlorine, bromine, hypochlorites, and hypobromites decompose solutions of urea, with evolution of nitrogen.

A compound of urea with sodium chloride, of the formula CH_4ON_2 , $\text{NaCl} \cdot \text{H}_2\text{O}$, separates in brilliant rhombic crystals when mixed solutions of urea and common salt are evaporated. This compound sometimes crystallises from concentrated human urine

Salts of Urea

Urea has somewhat feeble basic properties. It forms a well-defined series of salts, all of which are more or less soluble. Many of them are decomposed (or hydrolysed) by excess of water, and the aqueous solutions are in all cases acid to litmus as the result of this hydrolytic dissociation. The nitrate and oxalate of urea crystallise well, and are employed for the isolation and detection of urea. Urea also combines with metallic salts, the compounds being mostly soluble, with the exception of those with mercuric nitrate.

Urea nitrate, $\text{CH}_4\text{ON}_2\cdot\text{HNO}_3$, separates in crystals when moderately strong nitric acid is added to a concentrated aqueous solution of urea, and the liquid cooled. The compound forms brilliant white scales or plates, or, if the deposition is slow, prismatic crystals. When nitric acid and urea are brought together on a microscope slide, and the reaction observed under a low power, the formation of obtuse rhombic octahedra is first noticed, the angles being constantly 82° . These octahedra change to rhombic and hexagonal tables, either separate or superposed (see Fig. 16, *a*), but also having angles of 82° . For the formation of nitrate of urea from normal

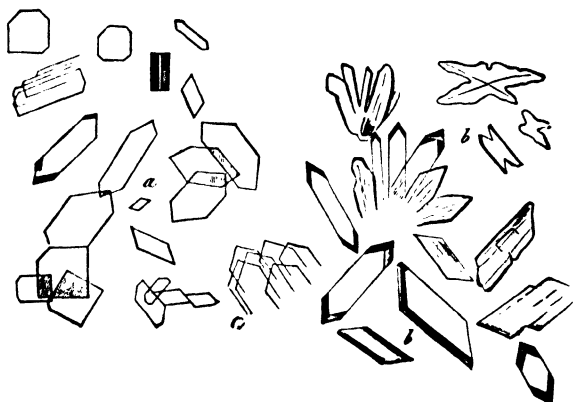


FIG. 17.—*a*, Urea nitrate; *b*, urea oxalate.

urine, it is sufficient to concentrate the liquid to about one-fourth of its volume, filter after cooling from the precipitated urates, etc., and to add nitric acid to the cold filtrate. Nitrate of urea is not changed in the air. It is readily soluble in water, forming a solution of acid reaction and taste. It is also soluble in alcohol, but very slightly soluble in presence of nitric acid. Oxalic acid precipitates urea oxalate from concentrated solutions of the nitrate.

Urea oxalate, $(\text{CH}_4\text{ON}_2)_2\cdot\text{C}_2\text{H}_2\text{O}_4$, is readily formed on mixing concentrated solutions of urea and oxalic acid. From urine it may be prepared by adding oxalic acid to the concentrated and filtered liquid. Urea oxalate forms thin crystalline plates (see Fig. 16, *b*), usually grouped together, but sometimes in well-formed separate crystals. Its microscopic appearance is not unlike that of the nitrate of urea, but the forms are less characteristic, and the angles are different. Oxalate of urea is soluble with difficulty in cold water,

but dissolves readily at the boiling point. It is less soluble in a solution of oxalic acid than in pure water. The salt dissolves in 62 parts of alcohol, but is quite insoluble in amyl alcohol. Hence, if a solution of urea in amyl alcohol (such as will result from evaporating urine to dryness, heating the residue with amyl alcohol, and filtering) be treated with a cold saturated solution of oxalic acid in amyl alcohol, urea oxalate will be precipitated in small crystals.

Urea hydrochloride, B, HCl , is a very deliquescent crystalline mass, formed by the action of hydrogen chloride on urea. It is decomposed by water into its constituents. $B, HAuCl_4 + 1 H_2O$ forms orange-red prisms or needles, very soluble in water, alcohol, and ether. $B_2, H_2PtCl_6, 2H_2O$ forms yellow needles, very soluble in hot water. B, H_3PO_4 is obtained in large, very soluble, rhombic crystals on evaporating pig's urine or mixed solutions of urea and phosphoric acid.

Detection of Urea

Urea produces no precipitate with tannin or other general reagents for the alkaloids. It gives no reaction with either neutral or basic lead acetate, and does not reduce Fehling's solution, even on boiling. It gives no colour reaction with oxidising agents.

If a fragment of solid urea is moistened with a concentrated solution of furfural, and a drop of strong hydrochloric acid (sp. gr. 1.10) then added, a fine violet coloration is produced (Schiff, *Ber.*, 10, 774).

If a residue containing urea is heated for some time to a temperature not exceeding 160° , the product will contain biuret. On dissolving it in water, adding sodium hydroxide, and then dropping in a dilute solution of cupric sulphate, a violet or red coloration will be produced if urea were originally present.

If an aqueous solution of urea is heated with silver nitrate, a white precipitate of silver cyanate is formed, soluble in boiling water, while the filtered liquid will be found to contain ammonium nitrate: $CO:N_2H_4 + AgNO_3 = AgOCN + NH_4NO_3$.

On mixing a solution of urea with one of neutral mercuric nitrate, a white flocculent precipitate is obtained. This has a composition dependent on the concentration of the liquid, containing, according to the conditions of its formation, 1, 1.5, or 2 molecules of mercuric oxide to 1 of urea. If, however, the addition of the mercuric

nitrate is continued as long as precipitation occurs, and sodium hydrogen carbonate is added in quantity sufficient to neutralise the nitric acid set free, the precipitate has the composition $\text{CH}_4\text{ON}_2, 2\text{HgO}$. The end of the action is indicated by the yellow colour developed from the formation of basic nitrate of mercury. The mercuric oxide compounds of urea are decomposed by hydrogen sulphide, with precipitation of mercuric sulphide and liberation of urea, and the procedure may be utilised for the isolation of the base from urine.

Urea is not precipitated by a solution of mercuric chloride. The addition of mercuric nitrate to a soluble chloride results potentially in the formation of mercuric chloride. As sodium chloride is present in urine, mercuric nitrate produces no precipitate in that liquid until sufficient has been added to react fully with the chloride present.

The recognition of urea in animal fluids is usually based on the preparation of the nitrate or oxalate. If the quantity of urea present is sufficient for the preparation of these salts in such amount as to allow a study of their properties, the determination of urea can be effected. On the other hand, if the quantity of urea present is very minute, as in the case of blood and of all secretions and excretions other than urine, it is not always easy to avoid error.

For the detection of urea in blood serum or other serous fluids, the liquid should be mixed with three or four volumes of alcohol, which precipitates the albuminous matters.¹ The filtered liquid is evaporated on the water-bath, and the residue exhausted with absolute alcohol. The alcoholic solution is evaporated on a watch-glass, and if foreign matters show themselves, the treatment with absolute alcohol is repeated. The extract is evaporated nearly to dryness on a watch-glass, the residue taken up with water, and any phosphates precipitated by addition of barium hydroxide solution. Carbon dioxide is passed through the filtered liquid, which is then boiled, again filtered, and evaporated on the water-bath to a syrup. The residue is divided into 2 or 3 portions, which are treated, respectively, with nitric acid and with oxalic acid, and the products examined under the microscope for the recognition of the characteristic crystalline forms of urea nitrate and oxalate.

¹ In some cases it is desirable to effect a preliminary separation of the bulk of the proteins by acidifying the liquid with acetic acid and boiling.

In carrying out the foregoing process, it is very important to study carefully the crystals supposed to be urea nitrate, and, whenever possible, to dissolve and test them with mercuric nitrate. Under certain conditions, and especially in presence of extractive matters, one may meet with nitrates of alkali metals which resemble in their microscopic appearance the crystals of urea nitrate. The inorganic salts are distinguished from the latter by their behaviour on ignition, and by the presence of a notable quantity of potassium or sodium in the ash, which will have an alkaline reaction. On the other hand, if a crystal of true urea nitrate is dissolved in water and treated with a concentrated solution of oxalic acid (which may be effected under the microscope), crystals of urea oxalate will be gradually formed.

The value of oxalic acid as a reagent for the isolation and recognition of urea is considerably enhanced if advantage be taken of the sparing solubility of urea oxalate in a mixture of alcohol and ether. A still better method is to heat the alcoholic extract to be tested with a small quantity of amyl alcohol, and then treat the solution, decanted or filtered if necessary, with a cold saturated solution of oxalic acid in amyl alcohol. The urea oxalate is precipitated in small crystals, which redissolve on warming the liquid, and on cooling separate out in a condition suitable for microscopic examination. The process may be modified by treating the solution of urea in amyl alcohol with one of oxalic acid in anhydrous ether. Precipitation takes place abundantly and quickly, but the crystals are usually small and imperfect. The oxalic acid may be added in powder, the liquid heated and thoroughly cooled, and the excess of oxalic acid removed from the precipitate by treatment with anhydrous ether. The method is capable of being employed quantitatively. The amyl alcohol used in the process must not develop a red or brown colour with oxalic acid, and should be free from water and ethyl alcohol.

Estimation of Urea

The estimation of the urea contained in urine is often of great physiological and pathological interest.

From 85 to 90% of the total nitrogen contained in normal human urine exists in the form of urea, the remainder being divided between ammonia, amino-acids, uric acid, hippuric acid, purine bases,

creatinine, etc. In the urine of herbivorous mammals the uric acid is replaced by hippuric acid, while the nitrogen of birds and reptiles is eliminated chiefly in the form of uric acid instead of as urea.

As urea is the predominant nitrogenous constituent of normal human urine, it is evident that for many purposes its estimation will afford sufficient information as to the amount of nitrogen passing away in the urine. There has been in the past much misconception as to the relations of urea to the other urinary nitrogenous compounds, and the older figures are relatively worthless. Urea represents the end-product (with ammonia) of the common metabolism of protein, both endogenous and exogenous; the eliminations of purines and of creatinine are independent, except that probably a small fraction of each is oxidised to urea. The relation of ammonia to urea in the urine is the expression of acidosis, *i. e.*, when acids are eliminated they withdraw ammonia from the conversion into urea. Since the urea represents the end-product of the metabolism of common protein, the urea output will vary with the protein of the diet. With a low protein diet, the urea output may fall to as low as 10 grm. per day, 60% of the nitrogen only being in the state of urea. With high intake of protein in the diet, the urea output may be as high as 75 or more grm. per day, or over 95% of the total nitrogen. Obviously, therefore, the relation of urea-N to total-N in the urine is of no value unless the protein intake is known, and the intestinal N-output as well. There is no purpose in making a urea estimation unless the nitrogen intake of the subject is accurately known and controlled, and the faecal nitrogen as well.

The amount of urea excreted in the urine varies considerably with the diet, being increased by nitrogenous foods. The weight of urea excreted per diem by an adult man on mixed diet ranges from 15 to 40 grm., the average being about 33 grm. (500 grains). On a diet poor in protein the excretion of urea may fall to 15 to 20 grm., whilst on a flesh diet the daily output may rise to 100 grm. The proportion of urea in human urine averages about 2%, but dog's urine is stated to contain 10%. This varies with the water output.

A large excretion of urea, if long continued, points to increased tissue metabolism or to surplus nitrogenous ingesta, but a temporary increase may be simply due to increased urination. Similarly,

diminished excretion of urea may be due to diminished metabolism or to retention of urea in the system.

A great number of methods have been devised at various times for the estimation of urea. The introduction of the enzyme urease, which occurs in very active form in soya beans, jack beans, etc., for its quantitative estimation has displaced the older methods, which were based upon the decomposition of urea into ammonia and carbon dioxide at higher temperatures and pressures under fixed conditions. The older methods, being only of historical interest now, will not be given.

The action of soya bean urease, for example, is quite specific for urea, ammonia and carbon dioxide being formed rapidly at ordinary temperatures, best at pH 7.0, whilst methyl urea and other substituted ureas are practically not decomposed at all. Various procedures have been described, differing in manipulative details, for carrying out the reaction practically. After the decomposition or hydrolysis of the urea the estimation of the ammonia may be carried out in the usual way, either by distillation into standard acid or by direct titration with a suitable indicator (methyl-orange). The method employed by Plimmer and Skelton (*Biochem. J.*, 1914, 8, 70) for the quantitative estimation of urea, and indirectly of allantoin, in urine is the simplest and easiest to carry out. It may best be described in their own words: "In its simplest features the method is no more than Folin's method (*Z. physiol. Chem.*, 37, 161) of estimating ammonia in urine. By fitting together three or four cylinders and Allihn bottles in series with a sulphuric acid bottle at the end, duplicate estimations of ammonia and urea in urine can be carried out simultaneously. In the cylinders for the urea estimations are put 50 to 60 c.c. of water, 1 gram. of finely ground soja beans and 5 (or 10) c.c. of urine. These cylinders are kept in a water-bath at a temperature of 35-40° and an air current is drawn through the series. After about an hour the rubber connections between the cylinders and bottles are disjointed and 1 gram. anhydrous sodium carbonate is dropped into the cylinders; they are then connected together again and the air current drawn through for another hour. To prevent frothing, liquid paraffin B. P. has been used (caprylic alcohol may also be used); it is superior to petroleum and toluene, as it does not evaporate, and it obviates the necessity of using a tube containing cotton wool between the

cylinder and Allihn bottle. It is not necessary to carry out a blank experiment with soya bean alone, since no ammonia was evolved by two different samples of the bean which were tested several times. The Allihn bottles are charged with excess of $N/10$ sulphuric acid (25 or 50 c.c.) which is titrated with $N/10$ alkali, using Alizarin Red as indicator . . .

"Not only urea, but also allantoin, is decomposed by the magnesium chloride method of Folin . . . Since urease has no action upon allantoin, the two substances can therefore be readily estimated in urines which contain both compounds; the difference between the two data will give the amount of allantoin."

The estimation of urea in other fluids may be carried out similarly. Several different methods have been described for the estimation of urea by means of the soya bean urease (E. K. Marshall, Jr., van Slyke, etc.), all based upon the same principle and differing only in some of the manipulations. Solid preparations from soya beans (or jack beans) containing the active enzyme are on the market under the names of Arlco-Urease and Urease-Dunning. The specific character of the soya bean urease also makes it a convenient reagent for getting rid of urea as such in solutions which are to be used for other purposes. As stated before, this method of estimating urea has replaced practically all of the former methods for most purposes.

A rapid method for the micro determination of urea in urine and blood by decomposing the urea with urease and measuring the volume of carbon dioxide produced in the manometric blood gas apparatus described by him has been given by Van Slyke (*J. Biol. Chem.*, 1927, **73**, 695). It is stated that micro determinations may be made with 0.2 c.c. of blood.

If the presence of urea is to be determined, the various qualitative tests which were described are useful. A more satisfactory reaction, which can be used for the direct gravimetric estimation of urea, has recently been described. This is the reaction with xanthyrol,

$\begin{array}{c} \text{C}_6\text{H}_4 \\ \diagup \quad \diagdown \\ \text{O} \end{array} \begin{array}{c} \diagdown \quad \diagup \\ \text{C}_6\text{H}_4 \end{array} \text{CHOH}$. If an alcoholic solution of xanthyrol is added to a dilute solution of urea in acetic acid, crystallised dixanthylurea,

$\begin{array}{c} \text{C}_6\text{H}_4 \\ \diagup \quad \diagdown \\ \text{O} \end{array} \begin{array}{c} \diagdown \quad \diagup \\ \text{C}_6\text{H}_4 \end{array} \text{CH.NH.CO.NH.CH} \begin{array}{c} \text{C}_6\text{H}_4 \\ \diagup \quad \diagdown \\ \text{O} \end{array} \begin{array}{c} \diagdown \quad \diagup \\ \text{C}_6\text{H}_4 \end{array}$ is precipitated. This

Dulcine forms colourless or yellowish shining needles, which melt at 173 to 174°. It is soluble in about 800 parts of cold, or in 50 of boiling water, and dissolves in 25 parts of rectified spirit. It is also soluble in ether and in benzene. When pure, it dissolves in concentrated sulphuric acid, without coloration. When boiled with water, dulcine gradually decomposes into ammonium carbonate and diparaphenetole carbamide.

On adding fuming nitric acid to a fragment of solid dulcine a violent reaction occurs, and the substance dissolves, with orange-red coloration. On evaporating the liquid to dryness at 100°, an orange-yellow resinous substance is obtained, which is soluble in ether, chloroform, or alcohol. If this resinous product is triturated with a mixture of equal parts of phenol and strong sulphuric acid the colour changes to a blood-red, which is permanent for a considerable time.

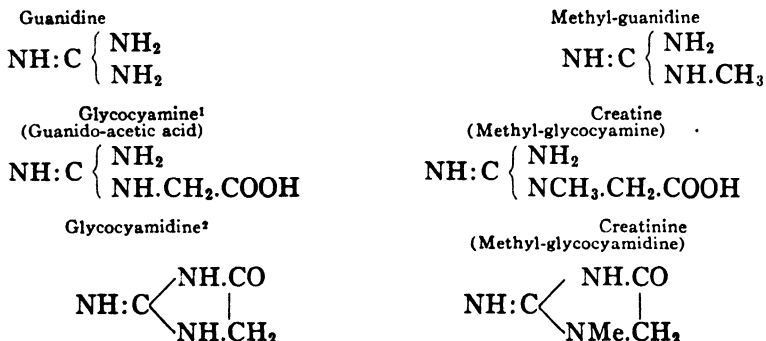
For the detection of dulcine in wine, beer, etc., G. Morpurgo treats the suspected liquid with 5% of lead carbonate, evaporates on a water-bath to a thick paste, and treats the residue several times with strong alcohol. The alcoholic solution is evaporated to dryness, and the residue extracted with ether. On evaporating the ether, a residue is obtained of nearly pure dulcine, which may be recognised by its sweet taste and by the following reaction:—The residue is warmed with 2 drops of phenol and 2 drops of concentrated sulphuric acid, the resulting reddish-brown syrup rinsed into a test-tube with a few c.c. of water, and the liquid cooled. Ammonia or sodium hydroxide is then poured cautiously on to the surface, when the production of a blue or violet-blue zone at the junction of the two layers will indicate the presence of dulcine.

Another test for dulcine in beer is the following:—The beer is treated with lime and evaporated to dryness. The residue is taken up with alcohol, poured into a saturated solution of sodium chloride, stirred, allowed to settle, and filtered. The filtrate, after evaporation to one-third its volume to remove the alcohol, is washed with petroleum spirit and extracted with ether. The ethereal extract is evaporated to dryness, recrystallised from alcohol, treated with a dilute solution of mercuric nitrate, heated on a water-bath for 15 minutes, and a small amount of lead dioxide added; the formation of a violet coloration indicates dulcine.

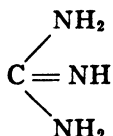
Dulcine may be separated from saccharin by extraction of the calcium salt with ether.

IMINO BASES

The imino bases are distinguished by containing the group —NH otherwise than as a link in a closed chain, as it exists in xanthine, uric acid, etc. In some cases, as in that of guanidine, the imino bases also contain one or more amino groups. The members of the group have in some cases considerable interest, and certain of them are important constituents of meat-juice and other animal products; but none of them has hitherto received any practical application in an isolated state. The chief members of the class may be thus formulated:



Guanidine. Iminourea



Guanidine has not been detected in any animal tissue or fluid, but has been isolated from vetch-seedlings. It has been obtained

¹ Glycocyanine is obtained when glycooll is boiled with guanidine carbonate in aqueous solution, ammonium carbonate being simultaneously formed. It forms transparent needles, sparingly soluble in cold water, but readily on boiling, and insoluble in alcohol. Boiled with cupric acetate it gives microscopic crystals containing $\text{Cu}(\text{C}_2\text{H}_4\text{O}_2\text{N}_3)_2$.

² Glycocyanidine hydrochloride is obtained when glycocyanine hydrochloride is heated to 160° . On boiling the product with water and lead hydroxide the free base is obtained in laminae of alkaline reaction, which have a bitter taste, are poisonous, and very soluble in water. Glycocyanidine forms a compound with zinc chloride which crystallises in needles closely resembling the corresponding compound of creatinine.

by the direct oxidation of proteins, and yields urea by boiling with barium hydroxide solution or dilute acids. Guanidine is the chief product of the action of oxidising agents on guanine, and may be regarded as a connecting link between creatine and the purine bases. While guanidine itself does not occur in the animal body, very important guanidine derivatives occur in the tissues and secretions.

Guanidine has been obtained synthetically by several methods, among which may be mentioned the reaction of cyanamide with ammonia: $\text{CN.NH}_2 + \text{NH}_3 = \text{NH:C(NH}_2)_2$. In practice, ammonium chloride is heated with an alcoholic solution of cyanamide.

Guanidine may be conveniently prepared by heating dry ammonium thiocyanate to $180\text{--}190^\circ$ for 20 hours. A portion of the thiocyanate is isomerised into thiourea, which reacts with the undecomposed ammonium thiocyanate to yield a product consisting mainly of guanidine thiocyanate. This is purified by crystallisation from water or alcohol, and the solution of 100 parts mixed with the solution of 58 parts of potassium carbonate, and evaporated to dryness. The potassium thiocyanate is removed from the residue by treatment with alcohol, and the guanidine carbonate recrystallised from water. From this salt free guanidine is obtained by dissolving it in the calculated quantity of dilute sulphuric acid and adding an equivalent amount of barium hydroxide solution. On evaporation *in vacuo* over sulphuric acid, the guanidine is obtained in deliquescent crystals.

Guanidine is a strongly alkaline, crystalline substance having a caustic taste. It is readily soluble in water and in alcohol. On exposure to air it deliquesces and absorbs carbon dioxide, with conversion into the carbonate.

When boiled with baryta water, guanidine yields ammonia and urea, thus: $\text{NH:C(NH}_2)_2 + \text{H}_2\text{O} = \text{NH}_3 + \text{CO(NH}_2)_2$. The urea further splits up into ammonia and carbon dioxide. With hot concentrated acids and alkalis these are the sole products.

Guanidine is a monovalent base which forms a series of crystallisable salts with acids. Guanidine nitrate, $\text{CH}_5\text{N}_3\text{HNO}_3$, forms crystalline plates, melting at 214° , which are sparingly soluble in water. The platinichloride $\text{B}_2\text{H}_2\text{PtCl}_6$ is sparingly soluble in absolute alcohol.

Many salts of guanidine, including the nitrate, sulphate, carbonate, and hydrochloride, give with Nessler's reagent a white or faintly yellowish precipitate, at first flocculent and bulky, but collecting together after a time. E. Schulze (*Ber.*, 1892, **25**, 661) describes the reaction as very delicate; a 0.05% solution gives an appreciable precipitate, while even a 0.01% solution is rendered turbid.

Guanidine is not precipitated by lead acetate, but is separated very completely by phosphotungstic acid, which reagent is employed by E. Schulze for its isolation from plant-substances.¹

Guanidine may be conveniently purified and estimated by precipitating the solution of one of its salts by an aqueous solution of picric acid. Guanidine picrate requires 2,600 parts of cold water for solution, and is only sparingly soluble in alcohol and in ether. It crystallises in very characteristic forms, either as needles or plates, m. p. 318–323°. It is insoluble in dilute sodium hydroxide solution and can be separated from creatinine in this way (Greenwald).

The picrolonate is soluble in an excess of alcoholic picronic acid solution, and can be separated from arginine in this way. With aqueous picronic acid an amorphous precipitate is obtained which forms clusters of thin needles when crystallised from hot water; m. p. 272°–274°.

With alcoholic cadmium chloride, a double salt, $\text{CH}_5\text{N}_3\text{HCl} \cdot 2\text{CdCl}_2$, m. p. 390–395°, is obtained.

Guanidine aurichloride, $\text{CH}_5\text{N}_3\text{HAuCl}_4$, forms long yellow needles difficultly soluble in water.

Guanidine has markedly poisonous properties. In dogs it produces paralysis, convulsions, vomiting, and difficult breathing; in frogs, muscular twitchings, paralysis, and (with a dose of 0.050 grm.) death.

Methylguanidine, $\text{NH}:\text{C}(\text{NH}_2).\text{NH}(\text{CH}_3)$, is produced by treating creatine with mercuric oxide, or with lead dioxide and dilute sulphuric acid.

Methylguanidine forms a strongly alkaline, deliquescent, crystalline mass, which evolves ammonia and methylamine when boiled

¹ Vetch-seedlings which had grown for 3 weeks in the dark were dried, powdered, and digested with rectified spirit. The extract was filtered, distilled, the residue treated with water and some tannin, and then precipitated with lead acetate. The filtered liquid was precipitated with phosphotungstic acid, the precipitate washed with dilute sulphuric acid, and decomposed with cold lime-water. The filtered liquid was freed from lime by means of carbon dioxide, and the filtrate neutralised with nitric acid, and concentrated on the water-bath. On cooling, guanidine nitrate crystallised out, 1 grm. being obtained from 3 kgrm. of vetch-seedlings.

with alkali hydroxide. B, HCl crystallises in needles insoluble in alcohol. $B, HAuCl_4$ forms rhombic crystals, melting at 198° , very sparingly soluble in cold water, but readily dissolved by alcohol or ether. Methylguanidine picrate, when first precipitated, forms a resinous mass, which, by boiling with water, is converted into needles melting at 197° and soluble in boiling absolute alcohol. It is insoluble in dilute $NaOH$ solution.

The presence of methylguanidine (as well as of guanidine and of dimethylguanidine) has been assumed in normal urine and in various decomposing animal matters. Although there can be no question that the guanidine picrates, etc., were isolated as such, the recent work of Baumann and Ingvaldsen (*J. Biol. Chem.*, 1918, **35**, 277) and of Greenwald (*J. Amer. Chem. Soc.*, 1919, **41**, 1109) showed that the methods used to separate these substances, including treatment with mercury or silver salts under certain conditions, involved the possibility of their formation by oxidation from creatine, methylguanido-glyoxylic acid, $CH_3NH.C(:NH)NH.CO.COOH$, being an intermediate product in the oxidation. It is therefore advisable in the procedures devised for the separation and identification of methylguanidine, for example, that no mercury or silver salts should be used. This fact obviously complicates the methods which may be used. The older work which apparently demonstrated the occurrence of the guanidines as such must therefore be discarded, or, at least, repeated with the use of the necessary precautions.

A method for the detection of the guanidines in urine has been developed by Greenwald, as follows:—The urine (1,000 to 1,800 c.c.) was first treated with a urease solution prepared according to Folin and Youngburg (*J. Biol. Chem.*, 1919, **38**, 111). About 1 gm. of jack bean meal was used for each 300 c.c. of urine. The mixture was kept at a temperature between 40° and 50° , and acetic acid was added as required to keep the reaction acid to litmus. The decomposition of the urea was apparently complete in 5 or 6 hours, but the mixture was allowed to stand at room temperature overnight. It was then treated with an excess of basic lead acetate solution, and then ammonium hydroxide was added until no further precipitation occurred. After standing about 30 minutes, the volume of the mixture was measured and it was filtered on a Buchner funnel. The volume of the filtrate was measured and it was then treated with

hydrogen sulphide. The precipitate was filtered off and washed. The filtrate and washings were evaporated under diminished pressure, with the outside bath temperature not over 40° , to a thin syrup. Concentrated hydrochloric acid was then added until the mixture was acid to Congo red, after which 10 c.c. in excess and about 500 c.c. of water were added. The evaporation, under reduced pressure, was repeated until a heavy crop of crystals appeared. This served to hydrolyse most of the very considerable amounts of acetamide that had previously been formed. The mixture was then treated with about 4 volumes of alcohol, filtered from the precipitated salts, which were washed with alcohol, and the evaporation and precipitation were repeated until there was practically no residue insoluble in absolute alcohol. After evaporating off the alcohol, again under diminished pressure, the residue was taken up in water and treated with a small volume of basic lead acetate solution and enough ammonium hydroxide to precipitate almost all the lead. The precipitate was filtered off and washed, the filtrate was freed from lead with hydrogen sulphide, and was then evaporated, under diminished pressure, to very small volume. After the addition of about 2 volumes of alcohol and then 2 drops of a saturated solution of zinc chloride in alcohol for every 100 c.c. of urine taken, the mixture was allowed to stand 2 days. The precipitated creatinine zinc chloride was filtered off and washed with a little alcohol. The filtrate and washings were diluted with about 4 volumes of water, made slightly alkaline with ammonia, and a few drops of lead acetate solution added. Under these conditions, the filtrate obtained after treatment with hydrogen sulphide was perfectly clear and contained no colloidal zinc sulphide, which was otherwise apt to be the case. The filtrate was acidified to Congo red with hydrochloric acid and evaporated, under diminished pressure, to small volume. The treatment with alcohol was repeated until all was soluble in absolute alcohol. The ethyl alcohol was then replaced by isopropyl alcohol and the material insoluble in this solvent also filtered off. Several volumes of water were added, and the evaporation was repeated to give a small volume. The liquid was filtered from a small quantity of oil and then treated with a concentrated solution of sodium picrate and enough sodium hydroxide to make the mixture alkaline and have an excess of about 0.1%. Guanidine, methylguanidine, and dimethylguanidine

picrates are precipitated under these conditions, but the small amount of creatinine remaining is not, for it enters into the well-known Jaffé reaction. After standing in the ice-box overnight, the crystals, if any were present, were filtered off and recrystallised from a little dilute acetic acid. They were then filtered off, dried, weighed, and analysed. The picric acid content was determined by solution in hot water and precipitation with nitron (M. Busch and G. Blume, *Z. angew. Chem.*, 1908, **21**, 354), the nitron picrate being filtered off on a Gooch crucible and weighed. The cooled filtrate was treated with a few drops of nitric acid, the nitron nitrate was filtered off, the filtrate was neutralised to litmus with sodium hydroxide, and a sufficient excess to give a concentration of about 10% in a volume of about 400 c.c. was added. This was distilled into standard acid until the volume had been reduced to about 130 c.c. and was then diluted and distilled again. If any considerable quantity of ammonia or amine was obtained in this second distillate, the residue was diluted and distilled again. The third distillate contained only traces of ammonia or amine. Under these conditions, guanidine, methylguanidine, and dimethylguanidine give off all their nitrogen as ammonia, methylamine, and dimethylamine. Creatinine would give off only little more than half of its nitrogen.

Although this procedure is somewhat troublesome, it is necessary in order to obtain reliable results, because of the possible decomposition of some of the other substances which are present. No picrates of the guanidine were obtained in a number of samples of human urine which were analysed in this way. The reliability of the method was proved by the recovery of most of the guanidines which were added to urines before analysis.

Greenwald (*J. Amer. Chem. Soc.*, 1919, **41**, 1109) developed an analogous method for the detection of the guanidines in meat:—The meat was extracted several times with boiling water, the extracts precipitated with basic lead acetate, the filtrate evaporated to small volume under diminished pressure and poured into double its volume of alcohol. After removal of the precipitate, the filtrate was treated with hydrogen sulphide, the lead sulphide removed, and creatinine separated from the filtrate by adding a small quantity of a saturated alcoholic solution of zinc chloride. The filtrate was treated with alcoholic mercuric chloride, filtered, treated with

hydrogen sulphide, filtered, evaporated to small volume, and precipitated with absolute alcohol. The filtrate was evaporated and precipitated again. This was repeated until the residue was practically entirely soluble in absolute alcohol. After dilution with water and boiling off the alcohol, the solution was treated with phosphotungstic and hydrochloric acids, the resulting precipitate decomposed with barium hydroxide, and the filtrate precipitated with alcohol as before. To the last alcohol-soluble precipitate, water was added, the alcohol boiled off, and sodium picrate added. The formation of a precipitate indicated a guanidine picrate which could be identified as already described.

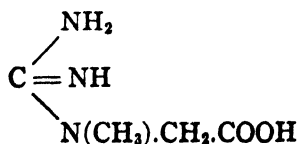
The results of Greenwald demonstrated that methylguanidine is not present in meat that is in a fair state of preservation. Whether or not it is present in badly decomposed meat has not been determined by the method outlined. This may depend upon the nature of the organisms responsible for the decomposition. It has been found that the creatine of meat is resistant to the action of the bacilli of the paratyphoid group, which is the type commonly found in "meat poisoning." In any event, methylguanidine can scarcely be regarded as playing an important part in the mechanism of "meat poisoning," for such poisoning is due to meat that is not badly decomposed.

Guanidine and methylguanidine hydrochlorides are soluble in absolute ethyl alcohol and in isopropyl alcohol. They may be separated from ammonium chloride and potassium chloride in this way. The picrates are insoluble in dilute sodium hydroxide solution, whilst creatinine picrate is soluble.

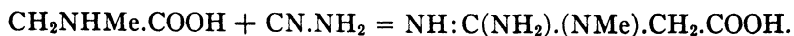
The melting point of α , α -dimethylguanidine picrate is given as 225° .

Methylguanidine has marked poisonous properties, the symptoms observed being rapid respiration, mydriasis, paralysis, convulsions, and death. Brieger found it to produce choleraic symptoms.

Creatine. Methyl-glyocyamine. Methyl-guanidine-acetic acid.



Creatine has been obtained synthetically by heating sarcosine with an alcoholic solution of cyanamide at 100° , thus:



It may also be obtained by heating sarcosine with guanidine carbonate to $140\text{--}160^{\circ}$.

Creatine is a constant constituent of muscle-substance, the flesh of fowls being said to contain 0.32%, cod-fish 0.17%, and beef 0.07%. But its isolation from these would not prove its pre-existence, since it is very readily formed by the hydration of creatinine, into which substance, on the other hand, creatine is very easily changed. Creatine exists pre-formed.

Creatine may be prepared from Liebig's extract of meat¹ which sometimes contains granular crystals of the base. The extract should be dissolved in about 20 parts of water, and the solution precipitated by a slight excess of basic lead acetate. The filtered liquid is treated with hydrogen sulphide, again filtered, and concentrated to a syrup at a moderate temperature, ebullition being avoided. Creatine crystallises out on standing in a cool place for some days. A more complete precipitation is effected if 2 or 3 volumes of alcohol are added. The precipitate of creatine is collected on a filter, washed with rectified spirit, and recrystallised from water.

A more satisfactory method of preparation is that developed by Benedict, which will be described in connection with creatinine.

Creatine is a white opaque substance, but it crystallises with one molecule of water in colourless, transparent monoclinic prisms (Fig. 18), which, when heated to 100° , lose their water and become opaque. Creatine is soluble to the extent of about 1% in water at 10° , and is very soluble in hot water. It is only very slightly soluble in absolute alcohol, more soluble in dilute spirit, and insoluble in ether.

On cooling a strong aqueous solution of creatine, the base separates in bulky needles. On more gradual evaporation of a dilute solution it is deposited in large prisms.

The aqueous solution of creatine has a slightly bitter taste, and is neutral to litmus. With acids, creatine reacts as a mono-acid

¹ Creatine was first isolated by Chevreul, in 1835, from a commercial meat extract of bouillon on which he was requested to report.

base, and combines to form crystallisable salts. The sulphate forms slender prisms, and the nitrate and hydrochloride thick short prisms. Creatine also unites with various neutral salts to form crystallisable compounds. That with zinc chloride forms small crystals, decomposed by water into its constituents. Creatine is not precipitated by lead acetate or phosphotungstic acid. When oxidised with permanganate and other oxidising agents (see methylguanidine), methylguanidine is formed. Oxidation with hydrogen peroxide in the presence of a little ferrous sulphide yields glyoxylic and formic acids, formaldehyde and methylguanidine. It does not combine additively with formaldehyde, as to the amino-acids. When heated with alkali, amines are formed.



FIG. 18.—Creatine.
(After Frey.)

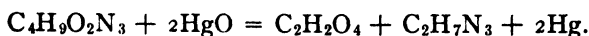
Conversion of creatine into creatinine, with loss of the elements of water, takes place when solutions of creatinine salts are heated. Also, on passing a current of hydrogen chloride over solid creatine, it is converted into creatinine hydrochloride. On evaporating a solution of creatine with the calculated quantity of dilute sulphuric acid, it yields creatinine sulphate. Conversion also occurs

very readily on boiling creatine with dilute hydrochloric acid, and the resulting creatinine can be readily identified by conversion into the zinc chloride compound. Creatine is also completely converted into creatinine by heating for 10 hours with dilute acetic acid. Only under certain conditions is this conversion into creatine quantitative. It is certainly accomplished by heating for 30 minutes with normal hydrochloric acid in an autoclave at 117° .

The reversible reaction $\text{creatine} \rightleftharpoons \text{creatinine}$ under different conditions of temperature and hydrogen ion concentration has been studied quantitatively by G. Edgar and H. E. Shiver (*J. Amer. Chem. Soc.*, 1925, **47**, 1179).

When boiled with aqueous barium hydroxide, creatine is decomposed into urea and sarcosine, methyl-hydantoin being also formed. Sarcosine and urea are also formed when creatine is heated in a sealed tube to 150° with an alkaline solution of barium chloride. The urea is further decomposed by the treatment, so that $2\text{NH}_3 + \text{CO}_2$ are formed from one molecule of creatine. By heating with

phosphoric acid to 150° , creatine yields methyl-hydantoin and one molecule of ammonia, whereas urea yields 2NH_3 under similar treatment. On heating an aqueous solution of creatine with mercuric oxide, oxalic acid and methyl-guanidine are formed:



Creatine does not precipitate a solution of zinc chloride, a behaviour which distinguishes it from creatinine.

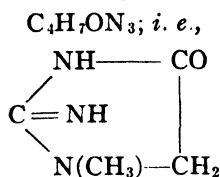
Creatine is also distinguished from creatinine by not being precipitated by a solution of phosphotungstic acid in presence of hydrochloric acid.

Creatine reduces Fehling's copper solution on long boiling, but no separation of cuprous oxide takes place. When heated with soda-lime, creatine yields methylamine.

Creatine may be estimated in the following manner:—In one sample of the urine or other material, an estimation of the creatinine is made according to the method of Folin (p. 364). Another sample is then heated for a half hour in an autoclave at 117° with *N*-hydrochloric acid, following which another estimation of creatinine is made. The difference represents the creatinine formed from creatine, and may be determined by direct calculation. If the solution contains sugar, this method is not applicable, on account of the colour due to the action of the acid upon the sugar. Rose has shown that by the use of phosphoric acid (half-normal) the conversion of creatine into creatinine may be accomplished and estimated in the presence of sugar.

Creatine occurs normally in the urine of infants and children. It occurs also under all conditions in which the body either has no sugar to burn or is unable to burn it; in starvation, diabetes, sepsis. It occurs also under circumstances of exaggerated wasting of muscle substance. On ingestion, creatinine is eliminated in part unchanged if the dose is large; otherwise it is, in small part, eliminated as creatinine, in greater part burned or decomposed in the intestine. On administration of glycocyamine, creatine appears in the urine. Though it is believed that the creatinine of the urine is derived from creatinine of the muscle, the conditions of transformation are not understood.

Creatinine. Methyl-glycoyamidine. Methyl-guanidine-acetic acid anhydride.



Creatinine is an anhydride of creatine, $\text{C}_4\text{H}_9\text{O}_2\text{N}_3$, and is produced from the latter substance with great facility. Creatinine occurs constantly in human urine, the amount varying from 1.4 to 2.2 gm. per diem. Creatinine has been found in sweat and in the muscles of fishes, but apparently does not exist pre-formed to any extent in mammalian muscles.

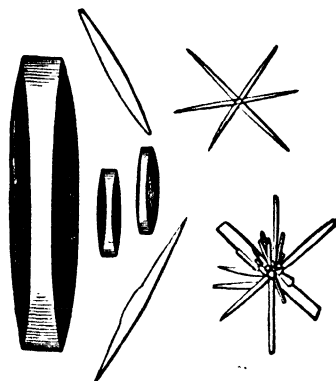


FIG. 19.—Creatinine. (After Prey.)

There are two fractions of creatinine in the urine of man—an exogenous fraction (due to the ingestion of creatinine-bearing flesh), and an endogenous fraction, derived from the metabolism of the muscular system. The endogenous creatine varies from 1.25 to 2.22 gm. per day, depending upon the amount of muscular tissue in the individual, of which it represents

the coefficient. It is an independent metabolism, and the endogenous creatinine is not dependent upon the total protein of the diet—it is a constant in each individual. It is not increased by muscular exercise, unless excessive or done in the untrained subject.

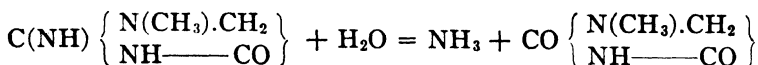
Because of lack of control of the endogenous creatinine, most of the reported observations bearing on the pathological variations in the urinary creatinine are not to be relied upon. In the acute stage of muscular dystrophies and degenerations, and in some fevers, the creatinine is increased. Late in the muscular dystrophies, it may be decreased. When creatine appears, the creatinine is correspondingly diminished, except in starvation.

As ordinarily obtained from urine, creatinine crystallises in oblique rhombic prisms and stellate forms (Fig. 18). It dissolves

in about 11 parts of cold water, and is sparingly soluble in alcohol, but insoluble in ether.

The aqueous solution of creatinine is neutral. The solution readily undergoes change, with formation of creatine, especially if ammonia, oxide of lead, or other base is present. By prolonged boiling with alkali hydroxide, creatinine is completely decomposed.

By boiling with baryta water, creatinine is hydrolysed to ammonia and methyl-hydantoin:



Boiled with water and mercuric oxide, it gives methylguanidine and oxalic acid (compare creatine). Heated with an alkaline solution of barium chloride, under pressure to 150°, creatinine behaves like creatine but is only partly decomposed by phosphoric anhydride at the same temperature.

Creatinine yields a series of crystallisable salts. The hydrochloride, B, HCl , crystallises in short transparent prisms from alcohol or in large laminae from water. It unites with zinc chloride to form the double salt $\text{ZnCl}_2, 2B\text{HCl}$. This is very soluble in water and alcohol, and must not be mistaken for the compound $\text{ZnCl}_2, 2\text{C}_4\text{H}_7\text{ON}_3$, which is one of the most characteristic salts of creatinine. Creatinine zinc chloride is obtained by mixing concentrated aqueous or alcoholic solutions of zinc chloride and creatinine, or by adding sodium acetate to the solution of the double hydrochloride. It forms oblique rhombic prisms or small needles, which have a tendency to form rosettes or warty concretions. The crystals are soluble in about 54 parts of cold or 27 of boiling water. They are insoluble in absolute alcohol, and require 9,200 parts of alcohol of 98%, or 5,700 of alcohol of 87% for their solution.

Mercuric chloride gives a white, curdy precipitate in strong solutions of creatinine, but the separation is not perfect unless sodium acetate is added, or mercuric acetate substituted for mercuric chloride. On allowing such a mixture to stand at the ordinary temperature, the compound is gradually deposited in microscopic spherules. The compound is almost insoluble in cold water, and is decomposed, with partial reduction of the mercury, by hot water. It is readily soluble in dilute hydrochloric acid, but is nearly insoluble in acetic acid.

From a concentrated solution of creatinine, silver nitrate precipitates crystals of the compound, $C_4H_7ON_3AgNO_3$. Mercuric nitrate does not precipitate a dilute solution of creatinine till excess of sodium carbonate is added, when $B_2Hg(NO_3)_2HgO$ is thrown down as a crystalline precipitate. Creatinine forms double salts with the chlorides of gold and platinum, and also a double salt with potassium picrate.

Creatinine possesses marked reducing properties. The mercury of the spherical salt, above described, is at once reduced, even in the cold, to the mercurous state and partly to metal on adding alkali hydroxide; contact with boiling water produces a similar change.

Creatinine reduces Fehling's solution on boiling, the blue liquid changing to yellow, but no cuprous oxide separates. Creatinine appears also to prevent the separation of a precipitate when dextrose is present, and hence exerts an interfering action on the application of Fehling's solution to the detection of dextrose in urine. Pavy's solution is reduced by creatinine without precipitation, and may be used for its estimation. Gold and silver are also reduced, but bismuth is not reduced in alkaline solution, a point of importance in the detection of sugar in the urine by the bismuth test.

Phosphomolybdic and phosphotungstic acids produce micro-crystalline precipitates in solutions of creatinine acidified with nitric or hydrochloric acid. By treating the precipitates with barium hydroxide, free creatinine is obtained.

The preparation of creatine and creatinine from urine in considerable quantities can be carried out by the process developed by Benedict (*J. Biol. Chem.*, 1914, 18, 183). The method is as follows:—To each 1,000 c.c. of urine (which must not be decomposed) 18 grm. of picric acid are added. It is not satisfactory to work with less than 10 litres of urine. The picric acid is dissolved in boiling alcohol (40 grm. to 100 c.c.) and the hot solution is added with stirring. The mixture is allowed to stand over-night, and the supernatant fluid is siphoned off. The residue is poured upon a large Buchner funnel, drained by suction, washed once or twice with cold saturated picric acid solution, and dried by suction. The dry, or nearly dry, picrate is treated in a large mortar or evaporating dish with enough concentrated hydrochloric acid to form a moderately thin paste (about 60 c.c. of acid for each 100 grm. pic-

rate), and the mixture thoroughly stirred with a pestle for 3 to 5 minutes. The mixture is then filtered by suction on a hardened paper, the residue washed twice with enough water to cover it, and sucked as nearly dry as possible each time. The filtrate is at once transferred to a large flask and neutralised with an excess of solid magnesium oxide added in small portions, the flask being cooled under running water. When all the hydrochloric acid has been neutralised the mixture will turn bright lemon yellow, or litmus paper may be used to test it. The mixture is then filtered by suction and the residue washed twice with water. The filtrate is at once strongly acidified with a few c.c. of glacial acetic acid, and (no attention being paid to a precipitate which may form at this point) the solution is diluted with about 4 volumes of 95% alcohol and filtered by suction at any time more than 15 minutes after a slight precipitate (chiefly calcium sulphate) has formed. The final filtrate is treated with 30% zinc chloride solution, 3-4 c.c. being used for each 1000 c.c. of urine originally used. This mixture, is stirred (a precipitate should form almost immediately) and allowed to stand overnight in a cool place. The supernatant fluid is then poured off and the precipitated creatinine zinc chloride collected on a Buchner funnel, washed once with water, then thoroughly with 50% alcohol, and finally with 95% alcohol and dried. The product should be a nearly white, light crystalline powder. 90 to 95% of the creatinine originally present should be recovered. Ordinarily 1.5 to 1.8 gm. of the double salt should be obtained per 1,000 c.c. of urine used. To prepare creatine from the double salt, 100 gm. of the latter are treated with 700 c.c. of water in a large casserole and heated to boiling. 150 gm. of pure calcium hydroxide are then added, with stirring, and the mixture boiled gently for 20 minutes (with occasional stirring). The hot mixture is filtered by suction, and the residue washed with hot water. The filtrate is treated with hydrogen sulphide gas for a few minutes and poured through a folded filter to remove the zinc. The filtrate is acidified with about 5 c.c. of glacial acetic acid and boiled down rapidly to a volume of about 200 c.c. This solution is allowed to stand over-night in a cool place. The crystallised creatine is filtered off with suction, washed with a very little cold water, and then thoroughly with alcohol and dried. (The filtrate obtained at this point should be diluted with alcohol and treated with zinc chloride

(50 c.c. of a 30% solution) for the recovery of unconverted creatinine.) This product is recrystallised by dissolving it in about seven times its weight of boiling water, and allowing the solution to cool slowly and then to stand for some hours. The crystallised product should be filtered off, washed with alcohol and ether, and dried in the air for about half an hour. Thus obtained, the creatine contains water of crystallisation which it loses very readily upon exposure to air. To prepare creatine which can be weighed with accuracy, it is necessary to dehydrate this product by heating it for some hours at about 95°. The yield in this process is about 18 grm. of recrystallised creatine and about 55 grm. of recovered creatinine zinc chloride. To prepare pure creatinine from the double salt, the latter, finely powdered, is placed in a dry flask and treated with seven times its weight (by volume) of concentrated aqueous ammonia. The mixture is warmed slightly and gently agitated until a clear solution is obtained, care being taken to drive off no more ammonia during the warming than is necessary to obtain a clear solution. The flask is stoppered, cooled, and placed in an ice-box for an hour or more. Pure creatinine crystallises out. The yield is 60–80% of the theoretical. If the product is coloured slightly yellow, it may be recrystallised either from boiling alcohol or by dissolving it in five times its weight (by volume) of concentrated ammonia (warming enough to effect solution) and letting the solution stand in a cold place for some hours. Recrystallisation is usually unnecessary.

For the quantitative estimation of creatinine by Folin's method (yellow to red colour with picric acid and alkali, an adaptation of Jaffé's reaction), it is now recommended to replace the dichromate solution formerly used as standard by a standard creatinine solution (Folin, *J. Biol. Chem.*, 1914, 17, 463). The creatinine zinc chloride double salt may be used and is obtained pure by three recrystallisations from 10 parts of boiling 25% acetic acid and addition of 1 part of a concentrated alcoholic zinc chloride solution and 1.5 parts of alcohol. 1.6106 grm. of the double salt, dissolved in 1,000 c.c. of *N*/10 hydrochloric acid gives a solution containing 1 mg. creatinine per c.c. Folin has given detailed directions (*J. Biol. Chem.*, 1914, 17, 469, 473) for carrying out the estimation of creatine and creatinine in urine, blood, milk, tissue, muscles, etc. For blood or milk the method is as follows:—10 c.c. of the fluid are placed

in a 50 c.c. glass-stoppered shaking cylinder, filled to the mark with saturated picric acid solution and shaken a few times. About 1 grm. of dry picric acid is added, and the mixture shaken for 5 minutes, transferred to centrifuge tubes, the sediment and precipitate shaken down, and the supernatant liquid poured through a filter. (If enough substance is available, the quantities taken may be doubled and filtered without preliminary centrifuging.) Proteins are removed by this treatment, and the creatine and creatinine obtained in the filtrate. The standard solution to be used contains 0.2 mg. creatinine per 100 c.c.; it is prepared by diluting the standard solution, described above, with saturated picric acid solution. 5 c.c. of 10% sodium hydroxide solution are added to both the unknown solution and 100 c.c. of the standard solution; the solutions are allowed to stand 10 minutes and compared in a Duboscq colorimeter. Neither solution may contain more than $1\frac{1}{2}$ times as much creatinine as the other, otherwise the comparisons of colour are not reliable. In collecting blood for this determination, 10 drops of a 20% potassium oxalate solution are sufficient to prevent clotting in 30 c.c. of blood. To estimate both creatine and creatinine in blood or milk, 10 c.c. of the filtrate from 10 c.c. of blood and picric acid solution are heated in an autoclave at 120° for 20 minutes in a flask covered with tin-foil, cooled, diluted to 25 c.c. with saturated picric acid solution, 1.25 c.c. of 10% sodium hydroxide solution added, and the colour compared with standard solutions containing 0.5, 1 and 2 mg. of creatinine in 100 c.c. of saturated picric acid solution, to 20 c.c. of each of which 1 c.c. of 10% sodium hydroxide solution has been added. With the standard set at 10 mm.,

$$\frac{10 \times 125 \times 0.5 \text{ (or 1 or 2)}}{\text{reading of unknown}} = \text{mg. creatine plus creatinine in}$$

100 c.c. blood or milk.

For the estimation of creatine and creatinine in the other substances similar methods are employed, with slight modifications in the procedure depending upon the properties of the material. For creatine plus creatinine in urine, a convenient method is the following (Benedict, *J. Biol. Chem.*, 1914, **18**, 192):—Such a volume of urine as will contain between 7 and 12 mg. of total creatinine is introduced into a small flask or beaker and from 10 to 20 c.c. of *N*-hydrochloric acid added, together with a pinch or two of powdered or granulated lead. The mixture is boiled over a free flame, as slowly or as rapidly

as may be desired, until very nearly down to dryness, when the heating should be continued to dryness either on the water-bath or by holding the vessel and heating carefully for a moment or two. The residue should preferably be left on the water-bath for a few minutes until most of the excess of hydrogen chloride has been expelled, after which it is dissolved in about 10 c.c. of hot water, and the solution rinsed quantitatively through a plug of cotton or glass wool (to remove all metallic lead) into a 500 c.c. volumetric flask. 20–25 c.c. of saturated picric acid solution are added and about 7–8 c.c. of 10% sodium hydroxide solution which contains 5% Rochelle salt (to prevent any formation or turbidity, due to dissolved lead—it has no effect upon the creatinine readings). The flask is filled to the mark at the end of 5 minutes and the colour comparisons made as usual. For creatinine in urine (Folin) 1 c.c. of the standard solution is measured into a 100 c.c. volumetric flask, 1 c.c. of urine into another, 20 c.c. of saturated picric acid added to each and then 1.5 c.c. of 10% sodium hydroxide solution. After 10 minutes the flasks are filled to the mark and the colour of the solutions compared. The special 1 c.c. pipettes, accurate to 0.1%, may be obtained from Eimer and Amend, New York. In estimating creatinine in urine, the colour due to sugar and picric acid appears too slowly to interfere, especially in the cold. β -Hydroxybutyric acid, at least in the amounts ordinarily present, does not interfere. Acetone and aceto-acetic acid interfere markedly and must be removed; acetone by aeration, aceto-acetic acid by extraction with ether and subsequent aeration, or by distillation *in vacuo* at temperatures below 65° in the presence of some phosphoric acid (Greenwald, *J. Biol. Chem.*, 1913, 14, 87). For creatine plus creatinine in diabetic urines, the autoclave method or evaporation to dryness cannot be used; Folin's original method of heating on the water-bath during 3–4 hours with hydrochloric acid is the only safe process; a little evaporation should be permitted, to remove acetone.

To estimate creatine and creatinine in muscle or other tissues, a preliminary treatment with picric acid solution removes the protein substances as insoluble picrates.

A more recent study by Greenwald (*J. Biol. Chem.*, 1924, 59, 601) has shown that the coloured compound formed in the Jaffé-Folin creatinine reaction is not due to the reduction of picric acid

to picramic acid or some similar compound, but is due to a tautomer of creatinine picrate which is transformed into ordinary creatinine picrate by heating to 139° . Anslow and King (*J. Chem. Soc.*, 1929, 1210) consider that an isomer is formed, and present structural formulas to account for the relations observed.

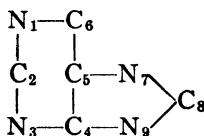
T. Weyl (*Ber.*, 1878, **11**, 228) has pointed out that, if a few drops of very dilute solution of sodium nitroprusside are added to a solution of creatinine, and dilute sodium hydroxide then added drop by drop, a fine ruby-red colour will be produced, which in a few minutes changes to an intense straw-yellow. If the liquid is now acidified with acetic acid and warmed, it turns greenish and Prussian blue separates. Guareschi recommends that 10% solutions of nitroprusside and sodium hydroxide should be used. The reaction is best obtained by first adding sodium hydroxide, and then a few drops of a concentrated solution of the nitroprusside. The reaction is very delicate, and can be obtained with a solution containing 0.03% of pure creatinine, or with urine containing 0.066%. In applying the test to urine the absence of acetone should be insured by distilling off a portion, since that substance gives a ruby-red colour with Weyl's test, though no blue colour can be obtained on acidifying, acetic acid merely restoring the yellow colour to red. According to Guareschi, a red colour is also yielded by hydantoin, methylhydantoin, and other compounds containing the group $\text{N}.\text{CH}_2.\text{CO}.\text{N}$. Creatine gives no reaction with Weyl's test unless the liquid is first boiled with a dilute acid, so as to convert it into creatinine. In this manner, Weyl demonstrated the presence of creatine in milk (*Ber.*, 1878, **11**, 2175). Formaldehyde, benzoyl chloride, permanganate, hydrogen peroxide and strong acids act upon creatinine just as they do upon creatine. In ammonia creatinine is quite soluble; creatine, however, quite insoluble. When creatinine is heated in weak alkaline solution for an hour, it is converted into creatine, though the reaction cannot be relied upon for quantitative estimation.

PURINE BASES. PYRIMIDINE BASES

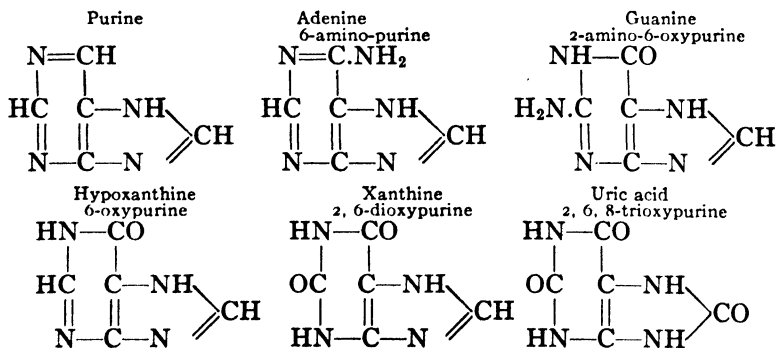
The purine bases are members of a most important group of substances concerned in both plant and animal physiology. Although their rôle in plant physiology is not so clear, in animal physiology the purines may be, in general, defined as the end-

products of the metabolism of nucleic acid, of the nuclei of cells. There is a regular wear-and-tear of cells in daily life, and cells die and are replaced by new cells. The nuclei of such degenerated and replaced cells undergo autolysis in the tissues in which they were resident, this being a reaction of digestion, a hydrolysis that splits the nuclein into protein and nucleic acid, and the latter into its three components, phosphoric acid, purine and pyrimidine bases, and pentose. It is the purine derived from this nucleic acid which constitutes the larger source of the purine bases of the urine. In the muscles a purine, hypoxanthine, is combined with a pentose in the protoplasm of the muscle cells outside the nuclei, being the only known extra-nucleic purine in the higher animal body, and from this a small fraction of hypoxanthine is daily derived. The purine metabolism is independent of the metabolism of common protein in the higher animals; in the birds and some reptiles the purines are the end-products of the common protein metabolism. But in the human metabolism, the purines are not so related.

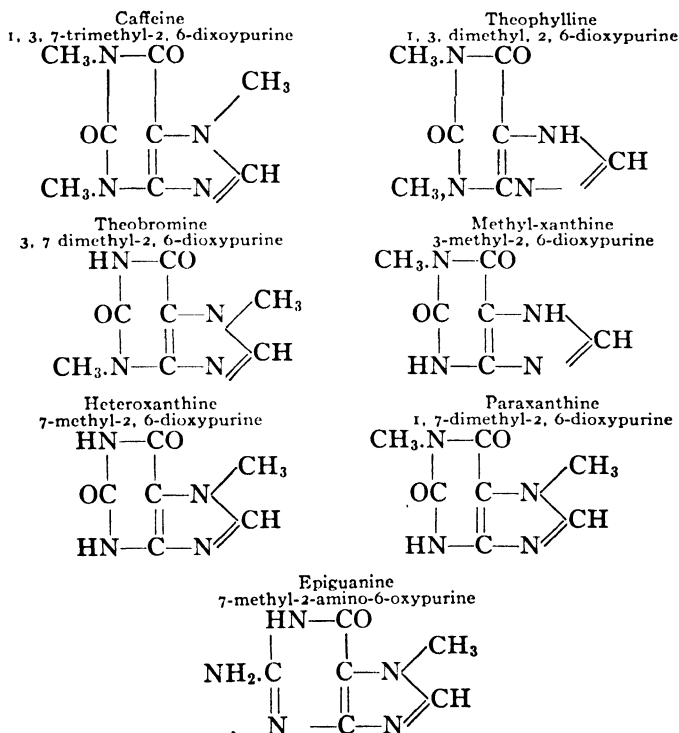
The purine bases are members of the group of purines, derivatives from the purine nucleus. This is to be regarded as a closed ring, and the different elements have, for convenience of nomenclature, been numbered.



The structures of the purine and the derivatives which occur in the animal body as the expressions of its endogenous metabolism are as follows:



In plants we have not only aminopurines, but also a special class, the methylpurines. These are present in many plants, but in particular in tea, coffee and cocoa. As a result, therefore, of the ingestion of these beverages, the methylpurines are introduced into human body. The body is able to demethylate these bases in part, but never completely, *i. e.*, at least one methyl group is always left to distinguish these purines from those derived from the nucleic catabolism. The structures of these methylpurines and of the methylpurines which are eliminated in the urine as the result of the ingestion of these beverages, are as follows:



From a contemplation of these structures, it is obvious that aminopurines are converted into oxypurines by deamination and oxidation, uric acid being the completed stage. The methylpurines are demethylated, but incompletely, one or two methyl groups being always left; complete oxidation to uric acid cannot be accomplished.

In all work and investigations with the purine bases the exogenous and the endogenous fractions must be carefully separated. A purine-free diet must be employed with all subjects in whom the estimation of the purines is to be attempted. Milk, eggs and rice constitute a practically purine-free diet. The glandular meats (sweetbreads) are most rich in purines, all flesh contains some, the seed vegetables (peas and beans) contain quite notable amounts; and tea, coffee and cocoa are very rich in them; malted liquors fairly rich also. In the tissues and urine of a subject on a purine-free diet, only uric acid, xanthine, hypoxanthine, adenine and guanine are to be found. All the methylpurines to be found in the urine are derived from beverages. In mixed urines of individuals on mixed diets, three-quarters of the purine bases are methylpurines derived from beverages. The purine output on a purine-free diet, as an index of the nucleic metabolism, has no relation to the metabolism of common protein and is also independent of the cretinine metabolism. The total purine nitrogen output per day in health on a purine-free diet will vary from 0.1 to 0.2 grm., of which from 5 to 10% may be in the form of purine bases.

In their chemical and physical characters the purine bases present resemblances to uric acid. They are mostly slightly soluble in cold water, and, except caffeine and theobromine, are insoluble in alcohol, ether, or chloroform. They all yield white precipitates with phosphomolybdic acid, mercuric chloride, and ammoniacal lead acetate; and guanine and adenine are very perfectly precipitated by picric acid.

A general reaction of the purine bases (including uric acid) is their precipitation from ammoniacal solutions by ammoniacal silver nitrate as a gelatinous compound of the base with silver oxide. The precipitates are usually insoluble in ammonia, unless concentrated and used in large excess, but to ensure complete precipitation excess should be avoided. On treating the precipitates with dilute nitric acid of 1.10 sp. gr. they are converted into compounds of the bases with silver nitrate. These compounds are well-defined crystallisable substances, insoluble in water, and, in the cases of hypoxanthine and adenine insoluble in nitric acid of the above strength, even on boiling; or, at any rate, crystallising out rapidly on cooling. Guanine and adenine, are stated by G. Salomon (*Z. physiol. Chem.*, 18, 207) to behave similarly. The compounds of xanthine, hetero-

xanthine, and paraxanthine remain in solution after cooling, which difference of behaviour permits of their separation from the bases previously mentioned. The bases are all completely reprecipitated as their silver-oxide compounds on neutralising the nitric acid solution with ammonia. Heteroxanthine and paraxanthine may be separated from xanthine by taking advantage of the limited solubility of their sodium salts in sodium hydroxide solution, and from each other by utilising the sparing solubility of the hydrochloride of heteroxanthine.

At the same time it must be pointed out that the solubility of mixtures of these bases is frequently different from that which might be expected from their individual solubilities; thus xanthine, which is difficultly soluble in water, is readily soluble in an aqueous solution containing guanine chloride.

Most of the purine bases are precipitated by cupric acetate, especially on heating. A still more perfect separation is effected by cuprous solutions, the precipitate consisting in each case of the cuprous salt of the derivative. These compounds may be obtained by treating the neutral solution with a mixture of cupric sulphate and sodium sulphite or thiosulphate, or by mixing the ammoniacal solution with Fehling's solution, heating to boiling, and gradually adding a solution of dextrose. Instead of cuprous oxide separating in the free state, it combines with the xanthine derivative to form a white insoluble compound. Hence it is evident that the presence of xanthine and its allies, including uric acid, may prevent the detection of sugar in urine by Fehling's test to an extent dependent on the amount of the interfering body present. The fact is of considerable practical importance when small quantities of sugar are to be sought for.

If, instead of using dextrose, the mixture of the alloxur-base with Fehling's solution is treated with hydroxylamine hydrochloride, reduction of the copper to the cuprous state occurs in the strongly alkaline solution and at the ordinary temperature. Treated in this way, *guanine* and *xanthine* give precipitates which are at first white, but rapidly become green by oxidation. *Heteroxanthine* and *paraxanthine* give similar white precipitates. The *uric acid* precipitate, cuprous urate, $\text{Cu}_2\text{O} \cdot \text{C}_5\text{H}_4\text{O}_3\text{N}_4$, is yellowish-white at first, but rapidly becomes greenish. The *adenine* and *hypoxanthine* precipitates are white. *Theobromine* and *caffeine* are the only xanthine derivatives which do not give precipitates with the above reagent.

M. Krüger (*Z. physiol. Chem.*, **18**, 351) finds that, by employment of cupric sulphate and sodium hydrogen sulphite, all the xanthinoid substances containing a substituted NH-group in the molecule are precipitated very perfectly from warm solutions as cuprous compounds. Theobromine constitutes a remarkable exception to this rule, being, like caffeine, creatine and creatinine, unaffected by Krüger's reagent. In carrying out the method Krüger slightly acidifies the liquid containing the xanthine bases with sulphuric acid. Sodium bisulphite is then added, and this is followed by cupric sulphate, when the precipitable bases are thrown down as gelatinous or flocculent white precipitates, which gradually become green or brown. In some cases the solution must be heated to ensure complete precipitation, but in others the reaction occurs perfectly in the cold. The precipitates dissolve readily in mineral acids, but only with difficulty in hot acetic acid. They are not altered by sodium hydroxide, but dissolve in ammonia in presence of air. They are readily decomposed by alkaline sulphides.

Uric acid is completely precipitated by Krüger's reagent, as also are adenine, methyladenine, hypoxanthine, and guanine. Dimethylhypoxanthine is not precipitated from warm solutions, but from cold concentrated solutions it separates in fine yellow needles.

If sodium thiosulphate (hyposulphite) is substituted for sodium bisulphite, the behaviour of the xanthinoid substances is somewhat different, apparently owing to the formation of compounds soluble in excess of the thiosulphate. Adenine is completely thrown down in

	Cupric sulphate, and	
	Sodium hydrogen sulphite	Sodium thiosulphate
Uric acid	Precipitated	Precipitated.
Adenine	Ppted.	Ppted.
Methyl-adenine	Ppted.	Ppted.
Hypoxanthine	Ppted.	Precipitated on warming only.
Guanine	Ppted.	Ppted.
Dimethyl-hypoxanthine	Ppted. only from cold concentrated solutions	Not ppted.
Theobromine	Not ppted.	Not ppted.
Caffeine	Not ppted.	Ppted.

cold solutions on standing, and methyladenine and guanine behave similarly, but hypoxanthine is not precipitated even from moderately strong solutions unless warmed. The table on p. 372 shows the general behaviour of the xanthinoid compounds with Krüger's reagents:

E. Salkowski (abst. *J. Chem. Soc.*, 1895, **II**, 538) precipitates 1,000 c.c. of urine with ammoniacal silver nitrate, after removing the phosphates by magnesia-mixture. The silver precipitate is suspended in water, decomposed with hydrogen sulphide, and the filtered liquid evaporated to dryness. The residue is treated with a little water containing from 2 to 3% of sulphuric acid, which dissolves the xanthine bases, leaving the uric acid practically insoluble. After 24 hours, the liquid is filtered, and the purine bases reprecipitated by ammoniacal silver nitrate, and estimated from the weight of silver in the precipitate. By this process Salkowski found the amount of bases to be about 8 to 10% of the uric acid and subject to few variations.

The proportion of purine derivatives (other than uric acid) ordinarily present in urine is extremely small, but there is reason to believe that, under circumstances not fully understood, their amount is much increased and may then be of pathological importance. For the actual isolation and separation of the purine bases, 5 to 10 gallons of urine should be treated by instalments of about 1 quart at a time with neutral lead acetate in powder, as long as a precipitate is produced. The liquid is filtered and sodium sulphate added as long as lead sulphate is thrown down, the liquid poured off from the precipitate, sodium bisulphite and copper sulphate added, and the liquid boiled. The precipitate, which contains the purine derivatives as cuprous salts, is filtered off, washed, dissolved in dilute nitric acid, and excess of ammonia added, followed by silver nitrate. The precipitate, consisting of the argentic oxide compounds of purine, is separated, suspended in hot acidified water, and decomposed with hydrogen sulphide, the resulting silver sulphide filtered off, and the filtrate concentrated. If, instead of the silver precipitate being decomposed with hydrogen sulphide, it is boiled with nitric acid of 1.10 sp. gr., the silver nitrate compounds of the hypoxanthine and adenine will crystallise out immediately on cooling, while those of xanthine, paraxanthine, and heteroxanthine will

remain in solution, and may be recovered as the silver oxide compounds by rendering the filtrate ammoniacal.

The following procedure, (*J. Biol. Chem.*, 1919, 37, 525), which was developed for the separation of various groups of constituents from flesh and from spoiled meat, may suitably be given in this place. The components studied were: (1) total nitrogen, (2) non-protein nitrogen, (3) ammonia nitrogen, (4) total creatinine nitrogen (creatinine plus creatinine), (5) purine nitrogen. In outline the procedure adopted is as follows:—The total nitrogen and ammoniacal nitrogen are first determined on a small portion. The protein substances are then coagulated in a large sample of the meat, and the non-protein substances separated from the coagulum by washing with water. Upon the water solution of the non-protein substances thus separated, determinations are made of nitrogen, purine nitrogen, total creatinine nitrogen, and ammoniacal nitrogen.

Total Nitrogen.—Total nitrogen is determined directly on a 1 gram. sample of the meat by the Kjeldahl-Arnold-Gunning method.

Ammonia. (a) *In Meat.*—1 to 3 gram. of uniformly hashed meat are weighed, placed in a large test-tube (8" × 1"), treated with 5 to 15 c.c. of water, enough pure sodium chloride to form a saturated solution, and 1.2 c.c. of 10 % sodium hydroxide solution, and aerated for 2 to 3 hours into 10 c.c. of 0.01 to 0.1N acid, the amount of acid depending upon the amount of ammonia present. The excess acid is titrated with standard alkali, a mixture of methyl red and methylene blue being used as indicator. (The use of this indicator mixture was suggested by Dr. E. M. Frankel, who stated that it was devised by Dr. Alonzo E. Taylor and Dr. Caspar W. Miller. It gives an exceedingly sharp colour-change with 0.01N reagents.)

(b) *In Filtrates Containing Non-protein Nitrogen.*—25 to 40 c.c. of the filtrate are treated with sufficient sodium chloride to form a saturated solution, 1.2 to 1.5 c.c. of 10% sodium hydroxide solution are added, and the mixture is aerated as in meat.

Separation of Protein from Non-protein Constituents.—The sample of meat (125 gram.) is finely hashed and added to 400 c.c. of boiling water in an aluminium pan with a lip. The meat is broken up by boiling from 1 to 4 minutes, and when fresh meat is used, about 100 c.c. of alumina cream are then added. (The alumina cream is prepared by boiling in an open pail a solution of filtered 8% aluminium acetate diluted with about six times its volume of water for

8 to 12 hours, the water being replaced as evaporation occurs. The suspension is then filtered through canvas bags.) In the case of badly decomposed meat, 250 c.c. of alumina cream are necessary, whilst meat in intermediate stages of decomposition is treated with an amount of alumina cream between these two limits. After the addition of the alumina cream, the mixture is again brought to the boiling point, stirred, heating continued for another half minute, and the mass then filtered through a large paper filter (15 inches). If the coagulation has been properly carried out, a clear, faintly yellow filtrate is obtained. The mixture is allowed to drain completely, and the contents of the filter paper are very carefully returned to the aluminium pan by means of a porcelain spoon. The meat, which has clumped together during the coagulation, is broken apart with the spoon into small bits. About 300 c.c. of water are added to the coagulum in the pan, the mixture heated to boiling, and filtered as before. This washing and scooping out is repeated three times, making four washings in all. Finally, the filter paper and contents are washed once with about 100 c.c. of hot water, and the filtrate and washings made up to 3,000 c.c. As the meat decomposes it becomes more difficult to remove protein hydrolysis products, and it is necessary to add a little alumina cream with each boiling. The meat also becomes very rubbery. If a perfectly clear filtrate is not obtained, it has to be heated to boiling and more alumina cream added.

Creatine Plus Creatinine.—Creatine *plus* creatinine is determined as follows on a portion of the non-protein nitrogen filtrate equivalent to 1 to 2 grm. of meat:—5 c.c. of *N*-hydrochloric acid solution are added to the portion of the filtrate, and the solution is evaporated to about 2 c.c. in a 50 c.c. conical flask on an electric stove. The flask is then covered with a watch-glass and heated at a low heat on the electric plate for 4 to 5 hours to convert the creatine into creatinine at a temperature just below boiling point. When cool, the acid is neutralised with 10% sodium hydroxide solution, with one drop of 0.1% methyl red solution as indicator. Immediately upon neutralisation 15 c.c. of a pure saturated picric acid solution and 3 c.c. of a 10% sodium hydroxide solution are added. The solution is then allowed to stand for 10 minutes to permit the colour to develop, diluted, and compared in a colorimeter with a standard creatinine zinc chloride solution.

*Purine Nitrogen.*¹—About two-thirds to three-fourths of the entire filtrate (usually 2,000 c.c.) are first evaporated to 50 c.c. under diminished pressure (temperature 40–45°). If necessary, caprylic alcohol is added from time to time to prevent frothing during this evaporation. The concentrated solution is carefully washed into a 250 c.c. beaker, the final volume being made up to 100 to 150 c.c., and the purines precipitated as the silver magnesium compounds. This is done by first making the solution distinctly alkaline with ammonium hydroxide. 10 c.c. of an ammonical silver nitrate solution are then added, this solution being prepared by dissolving 26 gm. of silver nitrate in a litre of water and adding ammonium hydroxide until the precipitated silver hydroxide redissolves. 10 c.c. of a 6% disodium phosphate solution are added at this point, and finally 5 c.c. of an ammoniacal magnesia mixture solution (100 gm. of magnesium chloride, 200 gm. of ammonium chloride, in 1 litre of water, with the addition of ammonium hydroxide until an excess of ammonia is present). Thorough stirring accompanies the addition of each reagent. The solution is allowed to stand 2 hours, filtered on a fluted paper, the precipitate washed four times with a little cold distilled water, and then washed back into the beaker in which the precipitation had been made. The precipitate, consisting of silver magnesium purine compounds, is then decomposed by adding 10 to 15 c.c. of 10% hydrochloric acid solution and allowed to stand in the dark over-night. The resulting purine hydrochlorides are filtered into a litre round-bottomed flask, contains several bits of cracked porcelain, made slightly alkaline with sodium hydroxide, then distinctly acid with acetic acid, and 10 to 15 c.c. of 10% acetic acid solution added in excess. The solution is then heated to boiling, and 10 c.c. of a 40% sodium bisulphite solution and 10 c.c. of a 10% copper sulphate solution successively added. A white precipitate forms which becomes brown on boiling. The boiling is continued for 3 to 5 minutes. The copper purine compounds are filtered while very hot on a fluted paper and washed thoroughly with hot water. The precipitate is then washed into a Kjeldahl flask and the nitrogen determined by the usual Kjeldahl procedure. It is necessary to add only 15 c.c. of sulphuric acid; no potassium sulphate is needed, as digestion proceeds rapidly. This method, providing two precipitations, the first in alkaline and the second in acid solution, gives pure

¹ Modification of the method of Krüger and Schmid, *Z. physiol. Chem.*, 1905, 48, 1.

compounds upon which to determine the nitrogen. If desired, the individual purines might be isolated from the mixture.

Total Nitrogen of Filtrate.—This is determined on a 50 c.c. portion of the filtrate by the Kjeldahl-Arnold-Gunning method.

The results obtained in the decomposition of meat brought about by various organisms are of interest and may be summarised as follows: The creatine-creatinine values change only slightly with *Bacillus proteus* and *Bacillus paratyphosus B*, but decrease markedly with *Bacillus coli communis*, *Bacillus enteritidis*, *Bacillus subtilis*, and *Streptococcus brevis*. The purine values decrease rapidly with *Bacillus coli communis* and *Bacillus enteritidis*. With *Bacillus proteus* and *Bacillus paratyphosus B* the purine nitrogen showed a more or less irregular (accidental) variation for the first action, followed by a small decrease. *Bacillus subtilis* and *Streptococcus brevis* gave a distinct final increase in the purine, indicating a synthesis. The ammonia increased in every case. It appears that bacteria exert certain selective actions on definite substances or groups of substances. While the data are not as yet quantitative enough for careful comparisons, it appears probable that more careful determinations in which the conditions are controlled more accurately and with media, wholly or in part synthetic, a method might be at hand for distinguishing various strains of bacteria.

Before proceeding with the consideration of the individual purine bases, the following general relations for the five purines ordinarily met with, namely, guanine, adenine, xanthine, hypoxanthine, and uric acid, may be given from which a separation adaptable to a particular problem may be devised.

Guanine may be separated from the other three bases because of its insolubility in ammonia.

Uric acid may be separated because of its insolubility in sulphuric acid.

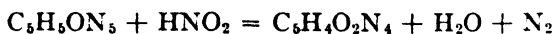
Adenine may be separated from hypoxanthine because of its difficultly soluble picrate.

Xanthine chloride is readily hydrolysed by water to form the insoluble free base; hypoxanthine chloride is soluble in water without decomposition.

Xanthine. $C_5H_4O_2N_4$. 2, 6-Dioxypurine.

The constitutional formula is given on page 368. Xanthine was originally discovered by Marcet (1819) in a urinary calculus, and

called "xanthine oxide." It has been found in Jarvis Island guano, occurs as a normal constituent of urine especially during the use of sulphur baths, and is present in minute quantities in yeast, the muscles of mammals and fishes, the liver, spleen, pancreas, thymus, brain, etc. It occurs also in very small quantities in plants, *e. g.*, in tea, malt-seedlings, lupins, etc. The only natural source from which it can be at all conveniently extracted is meat-extract. It has been produced synthetically by A. Gautier (*J. Chem. Soc.*, **48**, 275) by the reaction of hydrocyanic acid and water in presence of acetic acid, but its best mode of preparation is the decomposition of guanine by nitrous acid:



A nitro-compound is formed at the same time, which yields xanthine on reduction.¹

Pure xanthine forms a white powder consisting of microscopic crystals. It acquires a waxy lustre by friction. On heating xanthine a small portion sublimes unchanged, but by far the greater part chars, with evolution of cyanogen, hydrocyanic acid, carbon dioxide, and ammonia.

For the quantitative estimation of the purine bases in urine or tissue extracts, a double precipitation may be employed, *viz.* first precipitation with copper, lastly with silver. For the estimation of the bases in the urine, not less than 15 litres of urine suffice, and this is little enough. To the 15 litres of urine are added 300 c.c. of strong acetic acid. The urine should be free from albumin. The urine is brought to simmering, then 300 grm. of sodium acetate and 500 c.c. of a 40% solution of sodium bisulphite added, and then quickly about 750 c.c. of a 10% solution of copper sulphate, and the mixture kept simmering. The precipitate gradually turns brownish, and after ten minutes it is collected on a filter, washed thoroughly with hot water, then suspended in about 1 litre of hot water, thoroughly mixed and the copper precipitated by the addition of alkaline sodium sulphide, additional hydrogen sulphide gas passed through until precipitation of copper is complete, after which the mixture is acidified with acetic acid, boiled, and filtered hot. The filtrate

¹ According to E. Fischer (*Annalen*, **215**, 253) the best plan is to dissolve 10 grm. of guanine in a mixture of 20 grm. of concentrated sulphuric acid with 150 c.c. of water. After boiling, the liquid is cooled to about 80°, and 8 grm. of sodium nitrite added with constant agitation. The yield of xanthine is nearly quantitative; the product is of a pale orange colour, and free from the above mentioned nitro-compound.

should be clear. The collected filtrates are then acidified with hydrochloric acid and concentrated to about 50 c.c. and set aside for the crystallisation of the uric acid. On the following day, the uric acid is filtered off, washed with cold water three times and the collected filtrates used for the isolation and estimation of the purine bases.

If the attempt is to be made to separate the different purine bases, the method just described is the best to employ. It is, however, of no avail to make this attempt unless at least 50 litres of urine are worked up; otherwise enough of the bases will not be secured to make the isolation successful.

The solution of bases still contains a little uric acid, and this must be removed. The solution is first made alkaline, then acidified with acetic acid, heated to about 80° , and about 100 c.c. of a solution of permanganate added (a hot 0.5% solution of potassium permanganate is decolorised by the addition of alcohol, freshly prepared) than 10 c.c. of 10% acetic acid, and the mixture allowed to stand for a few moments. In this way the uric acid is removed by oxidation. The solution is then cooled, made strongly alkaline with ammonia, and a 10% silver solution added until the clear supernatant fluid shows an excess of silver when tested with nitric acid. The amount of ammonia must be enough to hold the silver chloride completely in solution. The gelatinous precipitate is collected upon a hardened filter paper, the precipitate washed with dilute ammonia until the wash water is free from chloride, then washed a couple of times with cold distilled water, suspended in water in a flask, magnesia added, and the mixture distilled until all fumes of ammonia have passed out. A fraction of this solution is then subjected to the Kjeldahl method for the estimation of nitrogen. The result is simply to be calculated as purine nitrogen. It is also possible to estimate the silver gravimetrically, or by titration with thiocyanate.

Xanthine is very sparingly soluble in water, requiring 1,400 parts of boiling, or 14,000 of cold water for solution. The hot aqueous solution deposits a pellicle on evaporation. Its reaction is neutral. In alcohol and ether xanthine is insoluble.

Xanthine dissolves with facility in solutions of sodium or potassium hydroxide, but is precipitated from the solution by adding an acid, even carbonic acid. It dissolves only slightly in dilute acids. It is dissolved by warm ammonia (distinction from uric acid),

and, on cooling, crystals of the ammonium salt separate; but on exposure to air, or on evaporating the solution, all the ammonia is lost and free xanthine remains.

The sodium salt, $\text{NaC}_5\text{H}_4\text{O}_2\text{N}_4 + \text{H}_2\text{O}$, crystallises in microscopic needles from a solution of xanthine in the smallest possible quantity of sodium hydroxide. Like hydrogen sodium urate, it is decomposed by repeated recrystallisations, and retains its water of crystallisation till heated to about 190° . On boiling xanthine with barium hydroxide solution, the sparingly soluble barium salt separates on cooling.

Xanthine hydrochloride, B_3HCl , is deposited in difficultly soluble glistening scales aggregated in nodules. The *sulphate*, $\text{B}_3\text{H}_2\text{SO}_4 +$

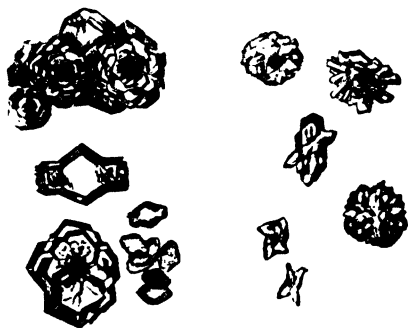


FIG. 20.—Xanthine nitrate. Xanthine hydrochloride. (After Kühne.)

H_2O , forms microscopic glistening tables, which lose the whole of their acid on washing with water. *Xanthine nitrate* forms fine yellow crystals of characteristic microscopic appearance (Fig. 20).

The *phosphotungstate* forms light straw-coloured cubical plates.

Mercuric chloride precipitates xanthine from very dilute solutions. A solution of 1 part

of xanthine in 30,000 gives a distinct opalescence with mercuric chloride.

Cupric acetate produces no precipitate in a cold solution of xanthine, but, on heating, a flocculent precipitate of apple-green colour is formed. With cuprous salts, or with Fehling's solution in presence of hydroxylamine hydrochloride (page 372), the compound $\text{Cu}_2\text{O}, \text{C}_5\text{H}_4\text{O}_2\text{N}_4$ is thrown down as a white precipitate, which rapidly turns green from oxidation.

An ammoniacal solution of xanthine gives precipitates with the chlorides of calcium and zinc, and with lead acetate.

An ammoniacal solution of xanthine gives a gelatinous precipitate of $\text{Ag}_2\text{O}, \text{C}_5\text{H}_4\text{O}_2\text{N}_4$ with ammoniacal silver nitrate. Treated with hot dilute nitric acid (sp. gr. 1.10) it dissolves, and the solution, after cooling, very slowly (if at all) deposits crystals of xanthine-

silver nitrate, $C_5H_4O_2N_4 \cdot AgNO_3$, grouped in a manner resembling wavellite. The same compound separates when a solution of xanthine in a minimum of nitric acid is treated with silver nitrate. Its greater solubility in hot nitric acid of the above strength distinguishes the silver compound of xanthine from those of hypoxanthine, carnine, adenine (and guanine); while those of (guanine) hypoxanthine and paraxanthine resemble the xanthine compound in their behaviour.

Xanthine gives the following colour reactions with oxidising agents:—

Strecker's Test.—Xanthine dissolves in hot nitric acid without evolution of gas. On careful evaporation of the solution a yellow residue remains, which turns reddish-yellow on addition of potassium or sodium hydroxide, and, on subsequent heating, becomes reddish-violet. If ammonia is substituted for the fixed alkali in the above test, no violet coloration is obtained. This behaviour distinguishes xanthine from uric acid, which gives the characteristic murexide reaction when similarly treated.

Weidel's Test.—If xanthine is treated with freshly-prepared chlorine water and a trace of nitric acid, and the liquid carefully evaporated to dryness, a residue is obtained which becomes pink or crimson on cautious exposure to ammoniacal vapours (compare uric acid).

Hoppe-Seyler's Test.—If solid xanthine is sprinkled on a solution of sodium hydroxide with which some bleaching powder has been mixed, each particle becomes surrounded with a dark green ring or scum, which rapidly becomes brown and disappears.

When xanthine is added to an alkaline solution of diazobenzene-sulphonic acid, a red colour develops.

When treated with hydrochloric acid and potassium chlorate, xanthine yields alloxan and urea.

Xanthine occurs in very rare cases as a urinary calculus. For its detection, the powdered calculus should be boiled with alkali hydroxide and the filtered solution treated with hydrochloric acid and again filtered. If xanthine is present in any quantity, hexagonal tables or globular masses of the hydrochloride will form as the liquid cools. The indication may be confirmed by dissolving the product in ammonia and adding ammoniacal silver nitrate, when gelatinous xanthine silver oxide will be precipitated.

If xanthine is brominated and then methylated, it will be converted into bromo-caffeine, and this is the preparation best adapted for the absolute demonstration of xanthine.

Heteroxanthine, $C_8H_8O_2N_4$, 7-monomethyl-xanthine, occurs in very small quantity in the urine of subjects consuming tea, coffee or cocoa. It forms needles, is soluble with difficulty in cold water, but much more readily on heating, and is insoluble in alcohol or ether. The crystals melt at 380° . The hydrochloride crystallises readily and is only sparingly soluble, which fact gives a means of separating heteroxanthine from the closely-allied base paraxanthine, the hydrochloride of which is more easily soluble. The two bases may be separated from co-occurring xanthine derivatives by the sparing solubility of their sodium salts in excess of sodium hydroxide.

Heteroxanthine gives an insoluble compound with ammoniacal silver nitrate. It is precipitated by copper sulphate with sodium bisulphate, by mercuric chloride, and by lead acetate and ammonia. It yields a brilliant colour with Weidel's test, but it gives no characteristic reaction with Strecker's test. It does not yield the colour test with diazo-benzene sulphonic acid.

Paraxanthine, 1, 7-dimethylxanthine.—This exists in minute quantity in urine after ingestion of tea, coffee or cocoa. Paraxanthine is sparingly soluble in cold water, but dissolves readily on warming, and is insoluble in alcohol and ether. It crystallises in flat, irregular, hexagonal tables when its solution is slowly evaporated, but, if the liquid is rapidly concentrated, the base separates in needles.

The silver nitrate compound of paraxanthine is soluble in hot dilute nitric acid, thus resembling the xanthine compound and differentiating paraxanthine from hypoxanthine, carnine, adenine, and guanine. Paraxanthine responds to Weidel's reaction, but gives no colour with Strecker's test, nor with diazo-benzene sulphonic acid.

Paraxanthine is further distinguished from xanthine by its greater solubility in water, and from heteroxanthine by the more ready solubility of its hydrochloride.

Guanine. 2-Amino-6-oxypurine. $C_5H_4ON_5$

Guanine may be prepared from Peruvian guano, which should be boiled with milk of lime till it assumes a greenish-yellowish colour,

when the liquid is filtered. The operation is repeated as long as a coloured filtrate is obtained. The residue, which contains the whole of the uric acid and guanine, is repeatedly extracted with sodium carbonate. The filtrate is treated with sodium acetate, hydrochloric acid added to strong acid reaction, and the guanine dissolved out of the precipitate by boiling it with dilute hydrochloric acid. The guanine hydrochloride, which separates on cooling, is separated from admixed uric acid by boiling it with dilute ammonia, and the residual guanine dissolved in hot concentrated nitric acid, which, on cooling deposits the nitrate, from which the free base may be liberated by ammonia.

Guanine and adenine may be readily prepared from nucleic acid as follows:—50 grm. of commercial yeast nucleic acid are heated at 100° for two hours with 200 c.c. of 10% sulphuric acid, concentrated ammonium hydroxide solution added slowly to the hot liquid until the neutral point is roughly reached, and then enough in sufficient excess to ensure that about 2% of ammonia is present. Guanine is

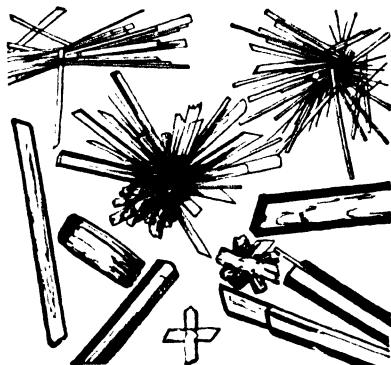


FIG. 21.—Guanine hydrochloride.
(After Kuhne.)

precipitated in heavy granular form, while adenine remains in solution. The guanine is washed with 1% ammonia, suspended in boiling water, and dissolved in the least possible amount of 20% sulphuric acid. After decolorising with charcoal and precipitating with ammonia, the guanine may be obtained perfectly pure by recrystallisation as the chloride, from which the pure guanine may be prepared with ammonia. The adenine is prepared from the guanine filtrates by precipitation as the adenine cuprous compound, washing the precipitate well, decomposing it with hydrogen sulphide, and crystallising as the sulphate (animal charcoal).

Guanine forms a white amorphous powder which may be heated to 200° without change. It is insoluble in water, alcohol, or ether. Guanine is distinguished from xanthine and hypoxanthine by its insolubility in hot dilute ammonia. It forms crystallisable salts with the stronger acids, guanine hydrochloride, $C_5H_4N_2O \cdot HCl +$

H_2O and guanine nitrate, $2B, \text{HNO}_3 + 3\text{H}_2\text{O}$, having characteristic microscopic appearance. The phosphotungstate forms straw-coloured needles or small cubical plates.

On adding a cold saturated aqueous solution of picric acid to a warm solution of guanine hydrochloride, guanine picrate, $B, \text{C}_6\text{H}_2(\text{NO}_2)_3\text{OH} + \text{H}_2\text{O}$, is thrown down as a highly insoluble precipitate of orange-yellow silky needles. Adenine is the only other base of the xanthine group which is precipitated by picric acid from dilute solutions, but guanidine gives a similar reaction.

Potassium dichromate throws down from solutions of guanine a highly insoluble, orange-red, crystalline precipitate of guanine dichromate, $B, \text{H}_2\text{Cr}_2\text{O}_7$. Potassium ferricyanide produces a brown, crystalline precipitate. Xanthine and hypoxanthine give no similar reactions. Guanine forms a platinichloride, but no aurichloride, by which again it may be distinguished from adenine. It forms a metaphosphate which is not soluble in excess of the reagent, whilst the metaphosphate of adenine is soluble. With ferrocyanide in solution with hydrochloric acid guanine forms a crystalline salt, in contradistinction to xanthine and hypoxanthine. The tests of Strecker and of Weidel give negative results, that with diazo-benzene sulphonic acid a positive reaction.

Guanine is converted into xanthine by treatment with nitrous acid (compare page 378). Bacteria and organic extracts effect the same deamination.

When boiled with strong hydrochloric acid, guanine is decomposed according to the equation:



Epiguanine. 7-Methyl-2-amino-6-oxypurine

Epiguanine is found in human urine following ingestion of tea or coffee. It is extremely insoluble. The crystals melt at 300° . It forms the usual insoluble combinations with silver nitrate and copper. The picrate is very insoluble; also the platinichloride and aurichloride. The Strecker and Weidel reactions are positive.

Hypoxanthine. 6-Oxypurine. $\text{C}_5\text{H}_4\text{ON}_4$

This base differs from xanthine by an atom of oxygen. It separates from its solutions as a white crystalline powder, difficultly

soluble in cold water, but soluble in 78 parts of boiling water, and in 900 parts of boiling alcohol. The base is insoluble in ether.

Hypoxanthine forms soluble crystallisable salts with acids. The microscopic appearances of the *nitrate* and *hydrochloride* are characteristic (Fig. 22). The urate, which is polymeric with xanthine, is precipitated on adding potassium urate to a solution of hypoxanthine hydrochloride.

The silver oxide compound of hypoxanthine, $\text{Ag}_2\text{O}\cdot\text{C}_5\text{H}_4\text{ON}_4$, is formed as a gelatinous precipitate on adding ammoniacal silver



FIG. 22.—Hypoxanthine nitrate. Hypoxanthine hydrochloride.

nitrate to an ammoniacal solution of the base. It is insoluble in ammonia, unless used in great excess, and it dissolves with difficulty in boiling nitric acid of 1.10 sp. gr. On cooling, a compound of the formula $\text{C}_5\text{H}_4\text{ON}_4\cdot\text{AgNO}_3$ separates in crystals, which, under the microscope, appear as long prisms or spindles, sometimes isolated, but in other cases crossed symmetrically to form stellate groups. The last form is common when the crystallisation occurs slowly. The characters of the silver nitrate compound allow of the separation of hypoxanthine from other bases of the group (see page 381). Hypoxanthine forms a crystalline *platinichloride*, but not an auri-chloride. The nitrate, while fairly soluble, is a very crystalline salt, and thus adapted to purification of the base. The *picrate* is

soluble in 500 parts of cold water, and forms long sharp needles. The phosphotungstate forms light straw-coloured cubical plates.

Hypoxanthine gives negative or only very faint reactions with Strecker's, Weidel's and Hoppe-Seyler's test (page 381). After treatment with hydrochloric acid and zinc it gives a ruby-red coloration on addition of sodium hydroxide in excess. In this reaction it behaves like adenine.

Hypoxanthine is almost always associated with xanthine. It occurs in the flesh and muscles of the heart of the horse and ox, in the pancreas, the spleen, and the liver, especially in cases of yellow atrophy. It has also been found in human and dog's urine. It may be isolated by the method described above and purified by solution in hot water, with addition of hydroxide of lead, filtration, separation of the lead as sulphide, and concentration of the filtrate to the crystallising point.

The reactions of Strecker and Weidel are negative, that of Kossel is positive. The Kossel test is made by heating a solution in a test tube for $\frac{1}{2}$ hour with zinc chloride and hydrochloric acid. The solution is first filtered, then made alkaline. The presence of hypoxanthine (also adenine, but not guanine and xanthine) leads to the development of a red colour, becoming brown on shaking.

Adenine. 6-Amino-purine. $C_5H_5N_9$

Adenine was originally obtained by Kossel when treating pancreas for the preparation of hypoxanthine, but is most conveniently prepared from yeast or from tea. Adenine bears the same relation to hypoxanthine that guanine does to xanthine (see page 368).

When pure, adenine crystallises from its aqueous solution in needles, which dissolve in 1,086 parts of cold water, and are readily soluble in hot water. It is but slightly soluble in hot alcohol, and is insoluble in ether. If crystals of adenine are slowly heated, it will sublime unchanged. But if the heating is done rapidly, it will melt at 365° , with evolution of gases and slight browning.

Adenine may be obtained in 4-sided pyramids, free from water of crystallisation, by adding excess of ammonia to a concentrated solution of its hydrochloride.

Adenine nucleotide may be prepared from tea leaves by extracting with dilute sodium hydroxide solution, precipitating with lead acetate, and recrystallising the brucine salt. W. S. Hoffman

(*J. Biol. Chem.*, 1925, **63**, 675) described the preparation of pure crystalline adenine nucleotide from pig blood and proved its identity with the adenine nucleotide from yeast nucleic acid (Jones and Kennedy) by its chemical composition and physical properties. Pig blood was deproteinised by heat in dilute acetic acid and sodium acetate solution, precipitated with lead acetate in faintly acid solution, the lead removed from the precipitate by means of hydrogen sulphide, evaporated to a syrup under diminished pressure, and the syrup hardened by means of absolute alcohol. The crude material was purified by recrystallisation of the brucine salt from 35% alcohol, and the crystalline adenine nucleotide recovered in the usual way through the lead salt.

Adenine yields crystallisable salts with acids, and also forms definite compounds with some neutral salts.

Adenine does not give the ordinary colour reactions characteristic of the xanthine bases, but resembles hypoxanthine in yielding a red coloration on treatment with hydrochloric acid and zinc with subsequent addition of an alkali.

An aqueous solution of adenine (0.5%) gives no precipitate with potassium ferrocyanide or ferricyanide until acetic acid is added, when thin crystalline plates are deposited. With chromic acid adenine forms the compound $(C_5H_5N_5)_2 \cdot H_2Cr_2O_7$, crystallising in 6-sided plates. Cupric sulphate produces in adenine solutions an amorphous greyish-blue precipitate, consisting of a mixture of copper-adenine and of adenine copper sulphate. Ferric chloride gives a red coloration unaltered by heat.

The phosphotungstate forms sulphur-yellow cubical or whetstone-shaped crystals.

The salt of copper and bisulphite is extremely insoluble. With metaphosphoric acid it forms a crystalline salt which is soluble in excess of the phosphate. The Strecker and the Weidel tests are negative, the Kossel and the diazo-benzene sulphonic acid tests are positive. The *oxalate*, the *silver nitrate*, the *picrate*, the *picrolonate*, and the *aurichloride* may all serve for isolation and identification. Nitrous acid converts adenine into hypoxanthine, in many ways a useful test.

Adenine and hypoxanthine combine in aqueous solution to form a compound containing $C_5H_5N_5, C_5H_4ON_4 + 3H_2O$, which crystallises from water in clusters of slender needles which readily effloresce, and

rapidly lose water at 100° . The compound forms a homogeneous hydrochloride, which may be separated into its constituents by dissolving it in dilute sulphuric acid and fractionally crystallising.

A method of obtaining adenine from molasses residues has been described by K. Andriik. (*Zeit. Zuckerind. Böhmen.*, **34**, 567). They are treated with copper sulphate and sodium hydroxide, the precipitate obtained being decomposed in suspension with hydrogen sulphide and filtered. The filtrate upon evaporation gives crystals of adenine which may be purified by recrystallisation (animal charcoal). 20 grm. of adenine were obtained from 40 kilos. of molasses residues.

For the separation of adenine from the allied bases, G Bruhns (*Ber.*, 1890, **23**, 225) employs the following process:—Silver nitrate is added to the nitric acid solution of the bases, when the silver nitrate compounds of the adenine and hypoxanthine are precipitated, and xanthine and guanine remain in solution. The precipitate is decomposed by hydrogen sulphide or dilute hydrochloric acid, the resulting solution nearly neutralised by sodium carbonate, and a solution of sodium picrate added. After standing 15 minutes, the precipitate of adenine picrate is filtered off, washed, dried at 100° , and weighed. The original precipitate contains $C_5H_6N_6, C_6H_2(NO_2)_3.OH + H_2O$, but becomes anhydrous at 100° , and melts, with decomposition, at 270° . Adenine picrate is soluble in 3,500 parts of cold water, so that a correction of 2.2 mg. must be made for every 100 c.c. of filtrate and wash-water. From the filtrate the hypoxanthine is precipitated by neutralising with ammonia and adding ammoniacal silver nitrate. No correction for solubility need be made if both solutions are free from excess of ammonia.

Adenine is very completely precipitated by cupric sulphate in presence of a reducing agent. By employing sodium thiosulphate as the reducing substance, and operating in a cold solution, separation from hypoxanthine can be effected.

Carnine. $C_7H_8O_3N_4$

Carnine crystallises in agglomerations of minute irregular crystals. It is very little soluble in cold water, but readily in hot, separating again on cooling. It is insoluble in alcohol or ether. Carnine forms crystallisable salts both with acids and bases. Its *hydrochloride* gives a golden-yellow precipitate with platinic chloride. The

compound with basic lead acetate dissolves in boiling water. The silver nitrate compound resembles that of hypoxanthine, and in its reaction with Weidel's test carnine also behaves like hypoxanthine.

Bromine water is decolorised when added to a boiling solution of carnine. On concentrating the liquid at 100° , brilliant needles of hypoxanthine *hydrobromide* are deposited, and, on treatment with caustic soda, yield the free base.

The occurrence of carnine in urine is doubtful. Hitherto it has only been found with certainty in extract of meat, of which it constitutes about 1%. Little is known about carnine. Indeed, its identity has been denied. It is probable that it is a complex purine, a fore-stage of adenine or guanine. It does not form a picrate, nor are the salts with silver and copper very insoluble; and in many ways it suggests the complex purines, since trimethylpurines have many of the same general reactions.

Methyl-xanthine. 1-Methyl-2-6-dioxy-purine

This is another of the methyl bases found in the urine after ingestion of tea or coffee. It forms fine white crystals. Crystallised from acetic acid, it forms 6-sided leaves. It is moderately soluble, and the alkali salts are very soluble. The *nitrate* and *hydrochloride* are not very insoluble, but crystallise well. It forms a platinichloride and an aurichloride. The usual insoluble salts with silver and copper occur. The Strecker and the Weidel tests give positive indications.

Isolation of Purine Bases

The purine bases may be isolated and separated, provided only that enough material is available. With 50 litres of urine a small amount can be separated; with several hundred litres better results are to be attained. For the isolation from tissue, this is hydrolysed with hydrochloric acid for several hours, and the filtrate employed for the manipulations.

The material is treated as far as the precipitation with copper and bisulphite, as previously described, and carried to the point of crystallisation of the uric acid. The filtrate from this crystallisation contains the purine bases. If it is very dark, it may be filtered hot with a little animal charcoal, but otherwise it is best to avoid this, as a loss is entailed. The solution is then evaporated to a syrup,

then taken up in alcohol, again evaporated to a syrup, and the process repeated. This is done to remove the hydrochloric acid. The final concentration is continued until a crumbly powder is obtained. This is then washed with cold water until free from hydrochloric acid, then with alcohol and ether. The insoluble fraction contains xanthine, heteroxanthine, guanine and methyl-xanthine; the solution contains adenine, guanine, hypoxanthine, and paraxanthine, with traces of heteroxanthine and methyl-xanthine. The exclusion of exogenous purines from the diet makes the situation much simpler, since then the fractionation is easy.

The bases in the xanthine fraction are dissolved in 3% hot sodium hydroxide solution, and set aside to cool. Within a day or two, the insoluble sodium salt of heteroxanthine crystallises out, and may be separated and tested as described under that heading. The filtrate is warmed to 60°, and to 6 parts are added a mixture of 2 parts of nitric acid in 2 parts of water. After a day or two, the crystals of xanthine nitrate will crystallise out. The crystals should be collected, dissolved in the least amount of alkali, then diluted to the original volume and treated again as before. The second crystallisation will usually be pure, and the collected crystals may be tested as previously described. To form the free base, the nitrate should be dissolved in water and evaporated while ammoniacal in reaction. The filtrate of the xanthine nitrate is made alkaline with ammonia, and the methyl-xanthine will then crystallise out on concentration. If search is to be made for guanine, this should be done with the first solution of the xanthine fraction. This is suspended in warm ammonia; on cooling, the guanine goes out of solution, all the others remain dissolved.

The bases in the soluble filtrate, the hypoxanthine fraction, are treated first by addition of ammonia until alkaline. After a day or two epiguanine (and possibly guanine) will crystallise out. The crystals are to be redissolved and treated with a very slight amount of ammonia, which will throw out epiguanine, but hold the guanine in solution, to be later recovered by strong ammonia. The two lots of crystals should be washed and tested as indicated under the respective headings. The filtrate from the epiguanine is freed from ammonia by heat, and adenine precipitated by careful addition of picric acid in 1% solution in slight excess. The picrate is filtered off at once, washed, and treated for identification as already indicated.

The filtrate is acidified with sulphuric acid, freed from picric acid by extraction with ether, and the bases then precipitated with copper and bisulphite, as previously described. The precipitate is freed from copper, and the filtrate concentrated, acidified with nitric acid up to 10% and set aside for crystallisation of the hypoxanthine nitrate, which is later collected and tested as previously described. In the filtrate are traces of paraxanthine, with possibly heteroxanthine and methylxanthine. As is obvious, the method is simple (and quantitative as well) in the absence of the methylpurines. And as the usual purposes of the test are to determine the metabolic purines, not the elimination of methylpurines, it is best to exclude all purine from the diet.

If one is estimating the methylpurines, it may be desirable to estimate the unaltered methylpurines that may be eliminated unchanged. Caffeine, theobromine and theophylline are not precipitated by the copper-bisulphite or the silver nitrate treatments. They are therefore contained in the original filtrates. The filtrate, concentrated if necessary, is acidified with sulphuric acid, and the bases precipitated with phosphotungstic acid. The precipitate is suspended in cold water, barium hydroxide added to the point of alkalinity, carbon dioxide passed through, and the mixture heated. It is filtered hot, and concentrated to a small volume. The residue is then extracted with chloroform, in which the caffeine is soluble, the others not. The theobromine is then precipitated as the double salt of silver nitrate, excess of ammonia being carefully avoided. The caffeine and theobromine may then be secured by crystallisation, in approximately quantitative manner.

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ANIMAL ACIDS

BY PHILIP B. HAWK, PH.D. AND OLAF BERGEIM, PH.D.

The acids occurring in the animal kingdom are in many instances (*e. g.*, oxalic, palmitic, benzoic) found also in plants, and a large proportion of them has been prepared by artificial means. Hence no sharp distinction can be drawn between substances of acid function found in animals and the organic acids from other sources, just as no sharp distinction can be drawn between animal bases and vegetable alkaloids. Many of the acids occurring in the animal kingdom have been already considered.¹ The acids belonging to the cyanogen group will be described in the sequel. There remain a limited number of substances of acid function, which, in their history and interest, are very closely associated with animal chemistry, and can be conveniently considered in this work under the heads of "Acids of Urine" and "Acids of Bile."

Acids of Urine

Of the acids existing in urine, whether in the free state or as salts, one of the most important is uric acid. Hippuric acid (page 425) exists in the urine of the herbivora, and ornithuric acid (page 427) in the urinary excrement of certain birds. Glycuronic acid is a urinary acid of considerable pathological interest. The "acetone bodies," including acetone, acetoacetic acid and β -hydroxybutyric acid, are considered in the present chapter (p. 435), as are also homogentisic acid and certain other aromatic hydroxy acids (p. 441). The oxyproteic acids are discussed on p. 444. Urine also contains various ethereal sulphates, but the more important of these have already been described.¹ Pyrocatechol and other phenolic substances, sometimes occurring in urine, were described in Volume III; while the simpler organic acids were considered in Volume I.

¹ Formic, acetic, butyric, valeric, oxalic, and succinic and lactic acids were described in Volume I. Palmitic, stearic, oleic, cerotic, and other of the higher fatty acids were considered in Volume II. For phenyl-sulphuric and benzoic acids, see Volume III. Indoxyl-sulphuric and skatoxyl-sulphuric acids are described on page 293 *et seq.*

Reaction of the Urine.—The mixed 24-hour urinary excretion of a normal individual ordinarily possesses an acid reaction to litmus. The actual hydrogen ion concentration varies over a wide range (pH 5.5–8.0), the mean being about pH 6. The reaction of the urine represents an equilibrium between a large number of acid and basic constituents, both organic and inorganic, which it contains. While organic acids and bases play a part, the reaction is ordinarily dependent, in the main, on the relative amounts of mono- and dibasic sodium and potassium phosphates present, the excretion of which by the kidneys is one of the factors in the regulation of the neutrality of the blood and of the organism in general. Normally the reaction depends mainly upon the predominance of acid-forming foods (chiefly high-protein foods) or of base-forming foods (vegetables and particularly acid fruits yielding carbonates in the body). Pathologically the acidity and organic acid content may be high, owing to the presence of excessive amounts of the acetone bodies which are found chiefly in diabetes.

Determination of Acidity by Titration (Folin).—Place 25 c.c. of urine in a 200 c.c. flask and add 15–20 grm. of finely pulverised potassium oxalate and 1–2 drops of 1% phenolphthalein. Shake 1–2 minutes and titrate with $N/10$ NaOH to a faint pink colour. Calculate the number of c.c. of $N/10$ NaOH required to neutralise the 24-hour specimen of urine. Normal figures range from 200–500, with an average of about 350.

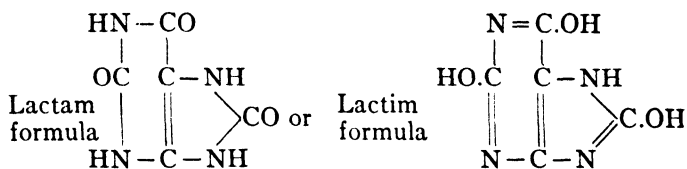
Determination of pH .—To 8 c.c. of boiled distilled water add 2 c.c. of urine and 10 drops of 0.04% water solutions of bromcresol purple, bromcresol green or phenol red. Compare with 10 c.c. portions of standard buffer solutions similarly treated in similar tubes, all at 38° C. To get the pH of undiluted urine subtract 0.1 pH .

Determination of organic acids (Van Slyke and Palmer). Mix 100 c.c. of urine with 2 grm. of finely powdered calcium hydroxide, stir occasionally for 15 minutes, and filter, to remove carbonates and phosphates. Transfer 25 c.c. of the filtrate to a 125–150 c.c. test tube, add 0.5 c.c. of 1% phenolphthalein and 0.2 N hydrochloric acid from a burette until the pink color just disappears. This amount need not be measured. The pH is now about 8. Add 5 c.c. of 0.02% tropaeolin OO, little by little, with stirring to prevent precipitation of the dye. Titrate with 0.2 N HCl until the red colour

is that of a standard made from 0.6 c.c. of 0.2*N* HCl, 5 c.c. of tropaeolin OO solution and water to make 60 c.c. When the end-point is nearly reached dilute the unknown to 60 c.c. Subtract from the number of c.c. of 0.2*N* HCl the amount (usually 0.7 c.c.) of the acid required to titrate a control tube of water between the same limits. Multiply the difference by 2 to get the amount of 0.1*N* HCl, and by 1,000/25 to get the value for 1,000 c.c. of urine. The titration includes organic acids, creatine, creatinine and a small amount of amino acids. Normally the excretion for 24 hours corresponds to about 8 c.c. of 0.1*N* HCl per kilo, of body weight. In diabetic acidosis values of from 20 to 180 c.c. have been observed.

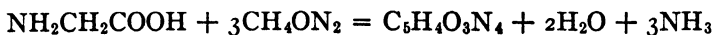
Foreman (*Biochem. J.*, 1928, **22**, 208), outlines rapid volumetric methods for the determination of amino acids, organic acids and bases in biological fluids.

Uric acid, 2-, 6-, 8-trioxypurine, $C_5H_4O_3N_4$.



Uric acid was discovered in 1776 by Scheele, who obtained it from urinary calculi; hence it was called lithic acid until Fourcroy changed the name to uric acid. It was found to be a constituent of the "chalk stone" deposits in the joints, by Pearson in 1798. Uric acid is one of the most constant and characteristic products of tissue metabolism in the human organism. In its formation the nucleic acids of the cell nuclei are especially concerned, uric acid arising from the oxidation of the purine bases contained therein. It occurs in the urine of all animals, in the excrements of birds, reptiles, scaly amphibians, snails and insects, as well as in other invertebrates. The white wings of certain butterflies have been shown, by Hopkins, to contain uric acid. Uric acid also occurs in the blood of birds, in human blood and in the bloods of other animals. Under pathological conditions it occurs in the blood in increased amounts in nephritis, in leucæmia, in gout, and sometimes also in arthritis. The so-called "chalk stones" and other gouty concretions commonly consist of the sparingly soluble hydrogen sodium urate, while the buff

coloured sediment which frequently separates from human urine usually consists of uric acid and urates of sodium or ammonium. Hydrogen ammonium urate constitutes the greater part of the urinary excrement of birds (guano), while that of serpents and other terrestrial reptiles contains it in a still purer form. On the other hand, uric acid is nearly absent from the urine of herbivorous animals, being replaced therein by hippuric acid. The synthesis of uric acid has been effected by Horbaczewski by heating glycoll with ten times its weight of urea to about 230° :



Behrend and Roosen also prepared it from *isodialuric* acid and urea, while E. Fischer and Tullner prepared it by boiling *isouric* acid with hydrochloric acid, and E. Fischer and Ach obtained uric acid by heating pseudo-uric acid with oxalic acid to 145° . The above formula shows that uric acid contains the residues of 2 molecules of urea, and explains the fact that the decompositions of uric acid almost invariably yield either a molecule of urea or some derivatives of urea, together with a second substance which can by further treatment be converted into urea. Many of the decomposition products of uric acid can indeed be prepared directly from urea. In view of the close relation existing between urea and uric acid, it is not surprising that the protein foods which in the mammal cause an increased secretion of urea, in birds give rise to uric acid.

Preparation.—Uric acid is best obtained by boiling serpents' excrement with dilute alkali hydroxide and treating the filtered liquid with excess of hydrochloric acid. On cooling, uric acid is deposited in a nearly pure state.¹ Guano may be boiled with a solution of 1 part of borax in 120 of water, and the filtered liquid precipitated with hydrochloric acid. Or it may be first treated with dilute hydrochloric acid to remove the phosphates, the residue boiled with dilute alkali hydroxide, and the filtered liquid treated with excess of hydrochloric acid. Another convenient source of uric acid is the yellowish deposit of acid urates formed on urinals. This may be boiled with sodium hydroxide as long as ammonia is evolved, carbon dioxide passed through the filtered liquid, and the precipitated

¹ For the isolation of uric acid from birds' excrement, the substance should be boiled with milk of lime as long as ammonia is evolved. The liquid is filtered boiling hot, when a filtrate is obtained not more highly coloured than urine, whereas sodium or potassium hydroxide gives a highly coloured liquid. From the solution the uric acid is precipitated by hydrochloric acid.

acid urate of sodium washed with cold water, dissolved in alkali hydroxide, and the solution decomposed with acetic acid. The product may be purified by dissolving it in hot alkali hydroxide, boiling with a little potassium permanganate or dichromate, and filtering the liquid into an excess of dilute hydrochloric acid. Uric acid may also be purified by solution in concentrated sulphuric acid and precipitation by addition of water.

Uric acid can be obtained from human urine, when free from albumin, by strongly acidifying the urine with hydrochloric acid and allowing it to stand in the cold for 24 hours. Dilute urines must first be concentrated before acidification. The precipitation of uric acid is not quantitative and may not occur under certain circumstances. The uric acid crystals are washed with cold water and alcohol to remove the pigments as well as benzoic and hippuric acids, which occur as contamination. The crystals can be further purified by dissolving them in a little sodium hydroxide and then precipitating as acid ammonium urate, by ammonium chloride, and decomposing this, after filtration, by hydrochloric acid; or the sodium urate solution is boiled with animal charcoal and then precipitated by hydrochloric acid. The uric acid can also be almost completely precipitated from the urine by an alcoholic solution of picric acid (25 c.c. of a 5% alcoholic solution of picric acid for 100 c.c. urine). This precipitate also contains *creatinine*. The washed and dried precipitate is boiled with dilute hydrochloric acid and the picric acid removed from the solution by shaking with ether, when the uric acid gradually precipitates.

Properties.—Uric acid when pure forms a crystalline, colourless powder, consisting of rhombic prisms or plates without taste or smell and of a sp. gr. ranging from 1.855 to 1.893. When precipitated from liquids containing urinary pigments or from extracts of guano, the crystals are always yellowish-red or brown, which colour is removed with difficulty by animal charcoal. In rapid crystallisation, small, thin, four-sided, apparently colourless, rhombic prisms are formed, which can be seen only by the aid of the microscope, and these sometimes appear as spools because of the rounding of their obtuse angles. The plates are sometimes six-sided, irregularly developed; in other cases they are rectangular with partly straight and partly jagged sides; and in other cases they show still more irregular forms, the so-called dumb-bells, etc. In slow crystallisation, as when the

urine deposits a sediment, or when treated with acid, large crystals separate which are coloured. Examined with the microscope these crystals always appear yellow or yellowish-brown in colour. The most common type is the whetstone shape, formed by the rounding-off of the obtuse angles of the rhombic plate. The whetstones are generally connected, two or more crossing each other. Besides these forms, rosettes of prismatic crystals, irregular crosses, brown-coloured, rough masses of broken-up crystals and prisms occur, as well as other forms. When deposited from urine or other impure solutions, dumb-bell, whetstone, and lozenge-like forms are among the most common and characteristic (page 416, Fig. 21, *b and c*). Garrod has shown that the pigments of urine are especially concerned in modifying the forms assumed by the uric acid, and that the presence of excess of one particular pigment will produce a corresponding definite variation in the form of the crystals. Edmunds has independently found that the forms assumed by uric acid greatly depend on the nature and amount of the coexisting substances. When precipitated from a solution of a pure urate by addition of hydrochloric acid, uric acid generally forms minute transparent rhombic plates (page 416, Fig. 21, *a*). Large crystals are obtainable much more readily from urine or other impure solutions than from pure urates.

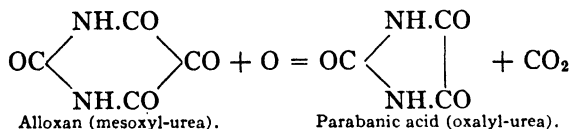
Uric acid is not volatile on heating, and dissolves at 18° in 39,480 parts water (His and Paul) and in 15,505 parts water at 37° (Gudzent). According to His and Paul 9.5% of the uric acid is dissociated in the saturated solution at 18° . Because of the reduction in the dissociation on the addition of strong acids, uric acid is soluble with difficulty in the presence of mineral acids. Uric acid is soluble in glycerin, but it is insoluble in alcohol or ether, slightly soluble in ammonia, but readily soluble in alkali hydroxides and in aqueous solutions of certain organic bases such as ethylamine, propylamine, diethylenediamine (piperazin), etc. Uric acid is soluble in neutral solutions of the borates, phosphates, hexamethylene tetramine, carbonates, acetates, and lactates of potassium and sodium, but not in solutions of the corresponding ammonium salts. In strong sulphuric acid, uric acid dissolves on warming, without decomposition, but when strongly heated with concentrated sulphuric acid, it is broken up and the nitrogen is entirely converted into ammonia. By phosphotungstic acid, in the presence of hydro-

chloric acid, uric acid is completely precipitated as a chocolate-brown precipitate, and it is slowly precipitated by basic lead acetate, and also completely precipitated by picric acid. The small amount of uric acid dissolved by ammoniacal solutions is not precipitated by ammoniacal silver nitrate, but a gelatinous, flocculent precipitate is obtained in the presence of salts of the alkalis or alkaline earths (magnesia). This precipitate is a double compound of uric acid with silver and these metals (Salkowski).

On being strongly heated uric acid decomposes, with the formation of urea, hydrocyanic acid, cyanuric acid and ammonia, and when fused with potassium hydroxide it yields potassium cyanide, potassium cyanate, potassium carbonate and oxalate. When heated with concentrated hydrochloric acid or hydriodic acid in a sealed tube to 170° it splits into glycoll, carbon dioxide, and ammonia.

By the action of oxidising agents on uric acid a number of compounds of great theoretical interest are obtainable which are mono- or diureides. These form two distinct series. The compounds of the first class, represented by alloxan, $C_4H_2O_4N_2$, are produced by acid-oxidising agents, such as nitric acid. Those of the second class, of which allantoin, $C_4H_6O_3N_4$, is the type, result from the oxidation of uric acid in alkaline or neutral solution.

By treatment with strong nitric acid in the cold, uric acid yields alloxan and urea: $C_5H_4O_3N_4 + H_2O + O = C_4H_2O_4N_2 + CO-(NH_2)_2$. Alloxan forms fine colourless crystals, very soluble in water and alcohol. The solution has an acid reaction, disagreeable astringent taste, and stains the skin red or purple after a time. Alloxan is decomposed by alkalis, and by oxidising and reducing agents. With ferrous sulphate, it gives a deep blue solution, precipitated on addition of an alkali. By further oxidation, alloxan is converted into parabanic acid, with evolution of carbon dioxide:



Parabanic acid is also produced by the direct treatment of uric with moderately strong and hot nitric acid. It forms colourless crystals readily soluble in water, forming a strongly acid liquid.

When heated with alkalis parabanic acid assimilates the elements of water and yields oxaluric acid.¹

Ammonium oxalurate is stated to exist in small quantity in human urine, from which it can be extracted by rendering a large volume (50 litres) of the liquid faintly alkaline to litmus, filtering from the resultant precipitate, and passing the clear liquid through a moderate quantity of animal charcoal. The charcoal is then washed with cold water till free from chlorides, dried at a gentle heat, and boiled with alcohol. The alcoholic liquid is filtered, evaporated on the water-bath, the residue exhausted with tepid water, and the brownish liquid evaporated to a syrup. On standing in the cold, ammonium oxalurate gradually separates in crystals, which should be washed with absolute alcohol, and recrystallised from boiling water.

If a drop of a solution of pure ammonium oxalurate is allowed to evaporate, the salt appears under the microscope in the form of long-pointed prisms, which reunite to form double hoops or rosettes. If the salt is impure, the hoops remain small and form globules armed with fine needles.

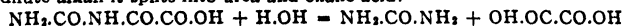
If a solution of ammonium oxalurate is treated with nitric acid, oxaluric acid crystallises out after a time dependent on the concentration. The crystals gradually disappear, and the liquid then contains urea nitrate, the characteristic crystalline forms of which can be observed by evaporating a drop and examining the residue under the microscope.

A moderately concentrated solution of ammonium oxalurate gives no precipitate with calcium chloride and ammonia, but on heating the liquid it becomes turbid, even before the boiling-point is reached, and ultimately an abundant precipitate of calcium oxalate is formed.

Ammonium oxalurate gives no immediate precipitate with silver nitrate, but after a time silver oxalurate separates in fine needles, which do not blacken in the light, and dissolve in ammonia to yield a solution which is not reduced by boiling.

Hot dilute nitric acid converts uric acid into alloxantin, $C_8H_4O_7N_4$, a substance which is also produced by the action of reducing agents on alloxan. It forms colourless crystals, soluble with difficulty in cold water, but more readily at 100° . The solution reddens litmus, gives with barium hydroxyde water a violet precipitate, which turns

¹ *Oxaluric acid*, $NH_2.CO.NH.CO.COOH$, forms a white crystalline powder of acid taste and reaction. It is but sparingly soluble in cold water. When boiled for some time with water or dilute alkali it splits into urea and oxalic acid:



white and disappears on heating, and reduces silver nitrate. Moistened with ammonia, or exposed to ammoniacal vapors, alloxantin yields a magnificent purple colour, due to the formation of ammonium purpurate or murexide, $(\text{NH}_4)\text{C}_8\text{H}_4\text{O}_6\text{N}_5$. The formation of this substance furnishes a delicate and characteristic test for uric acid (see page 404).

Chlorine and bromine convert uric acid at ordinary temperatures into urea and alloxan. On heating, parabanic and oxalic acids are also produced. Hypobromites and hypochlorites cause the evolution of a portion of the nitrogen of uric acid in a gaseous state, but the reaction does not appear sufficiently definite to serve as a means of estimating uric acid.

When oxidised in neutral or alkaline solution, by potassium permanganate or ferricyanide, lead peroxide, mercuric oxide or ozone, uric acid yields carbon dioxide and *allantoin*, $\text{C}_4\text{H}_6\text{O}_5\text{N}_4$.¹ By avoiding all rise of temperature, filtering, neutralising with acetic acid, and allowing the liquid to stand for 24 hours, crystals of allantoin are obtained in nearly theoretical proportion. Copper oxide with ammonia oxidises uric acid in the presence of potassium hydroxide to urea and oxalic acid.

Allantoin is the diureide of glyoxylic acid. It is the characteristic constituent of the allantoinic fluid, especially that of the calf, and is found in foetal urine and amniotic fluid. It is the chief end-product of purine metabolism in all mammals except man and the anthropoid apes, and occurs in very small amounts (5–15 mg. per day) in the urine of man also. It appears in the urine after internal administration of uric acid in dogs and other animals, and has been found in the young leaves of the plane-tree. Allantoin forms shining colourless prisms of characteristic microscopic appearance (Fig. 23). It is soluble in 160 parts of cold water, more readily in hot water or hot alcohol, but is insoluble in cold alcohol or in ether. Allantoin has a neutral reaction, combines with metallic oxides, and is soluble in solutions of alkaline carbonates. It reduces Fehling's solution on prolonged boiling and is precipitated by the nitrates of silver and mercury, in the presence of small quantities of ammonia, which reactions may be employed for its isolation. With furfural and hydrochloric acid it behaves like urea, but the coloration is less

¹ Allantoin, $\text{OC} \begin{cases} \text{NH} \cdot \text{CH} \cdot \text{HN} \cdot \text{CO} \cdot \text{NH}_2 \\ \text{NH} \cdot \text{CO} \end{cases}$

their solubilities. These investigations have also indicated that the first series (lactam salts) is unstable and at the moment of their formation in aqueous solution are changed into the second series (lactim salts) which are stable, a change which Gudzent explains by an intramolecular rearrangement, corresponding to the two isodynamic forms of uric acid. (Compare p. 395.)

Detection

Uric acid is commonly separated in the free state by adding excess of hydrochloric acid to its solution. When separated from urine in this manner, it forms a coloured deposit which adheres to the sides of the glass.¹ The best mode of operating is described later.

When isolated, uric acid is readily identified by its microscopic appearance, though the forms it assumes are very numerous (see page 417).

A highly characteristic and delicate reaction of uric acid is that known as the "murexide test," which is based on the behaviour of uric acid on oxidation. If uric acid, a urate, or even urine, is treated with a few drops of strong nitric acid, and the liquid evaporated to dryness in porcelain at 100°, a yellowish or red residue will be obtained, which owes its colour to the formation of alloxantin, $C_8H_4O_7N_4$. On inverting the capsule over another containing ammonia or otherwise subjecting the residue to ammoniacal vapours, it acquires a magnificent purple colour, owing to the formation of murexide or ammonium purpurate, $NH_4C_8H_4O_6N_5$. On now adding sodium hydroxide, the purple becomes changed to blue; the colour disappears on warming. An excess of ammonia or sodium hydroxide should be avoided. Somewhat analogous reactions are given by caffeine, theobromine, guanine, and xanthine, but the differences do not allow of their confusion with uric acid.

The nitric acid prescribed in the above test may be advantageously replaced by bromine water, or the material to be tested may be evaporated with a few drops of strong hydrochloric acid and a minute crystal of potassium chlorate.

A small quantity of uric acid is heated with dilute nitric acid until effervescence ceases, and then the excess of nitric acid is evaporated slowly until a coloration is obtained, and this residue is treated with 2 or 3 drops of concentrated sulphuric acid to which a few drops of

¹ A drop of fresh urine, mixed with hydrochloric acid, may be observed under the microscope to deposit uric acid crystals in the course of a few minutes.

commercial benzene (containing thiophene) have been added. A bluish coloration will be obtained, and this changes to brown on the evaporation of the benzene, but becomes blue again on the addition of benzene (Denigès' test).

On dissolving a small portion of uric acid in a little sodium hydroxide, filtering and treating the filtrate with an excess of ammonium chloride, a gelatinous precipitate of ammonium urate occurs immediately or after some time.

If a faintly alkaline solution of uric acid in water is treated with a soluble zinc salt a white precipitate forms which, when collected on a filter, gradually turns blue, owing to oxidation in the presence of sunlight. Potassium persulphate produces an immediate blue colour (Ganassini's reaction).

Uric acid is completely precipitated from its solutions by phosphotungstic acid in presence of hydrochloric acid.

If the uric acid is dissolved in a solution of sodium carbonate, and a drop of the liquid placed on filter paper previously moistened with silver nitrate, a yellow, brown, or black spot will be produced, owing to the fact that silver carbonate is reduced by uric acid even at the ordinary temperature.

On adding a little Fehling's solution to a solution of uric acid in sodium hydroxide, a greyish precipitate is formed, which is said to consist of cuprous urate; but with excess of the reagent, and on application of heat, red cuprous oxide separates and allantoin is formed. Uric acid does not reduce a hot alkaline solution of picric acid, nor does it reduce the Benedict or Nylander sugar reagents. This fact distinguishes it from creatinine, dextrose, and other normal and occasional constituents of urine which react with Fehling's solution.

If a small amount of the substance to be tested is dissolved in 20 c.c. of saturated sodium carbonate solution and 1 c.c. of Folin's phosphotungstic acid reagent is added, an intense blue colour will be obtained (see Estimation of Uric Acid).

For the detection of traces of uric acid in blood or similar liquids, from 150 to 300 c.c. should be boiled, so as to coagulate the proteins, and filtered. The filtrate is evaporated at 100°, and the residue completely exhausted with alcohol. The insoluble portion is then boiled with water, which will dissolve any urates present. The solution is cautiously concentrated, and acetic acid added in excess, when uric acid, if present, will gradually separate and can be recog-

nised by its microscopic characters and its reactions with nitric acid. The proteins of blood or other similar fluids may also be removed with tungstic acid (see Estimation of Uric Acid in Blood).

If the quantity of liquid available for the test is very small, 5 to 10 c.c. may be treated in a watch glass with 6 to 12 drops of strong acetic acid, and a cotton thread introduced. After standing for 24 hours in the cold, microscopic crystals of uric acid may be detected on the thread.

Estimation

Estimation in Urine.—The older methods for estimating uric acid in urine by precipitating it from its solution by the addition of hydrochloric acid and allowing it to stand, yield such inaccurate results that they are not used at the present time.

For determining uric acid in urine the colorimetric microchemical method of Benedict and Franke (*J. Biol. Chem.*, 1922, **52**, 387), is the simplest and is most commonly employed. The urine is diluted so that 10 c.c. will contain between 0.15 and 0.30 mg. of uric acid. Usually a dilution of 1:20 is satisfactory. Ten c.c. of the diluted urine are measured into a 50 c.c. volumetric flask, 5 c.c. of 5 % sodium cyanide¹ solution are added from a burette, followed by 1 c.c. of the arsenophosphotungstic acid reagent. The contents of the flask are mixed by gentle shaking and at the end of 5 minutes diluted to the 50 c.c. mark with distilled water and mixed. This blue solution is then compared in a colorimeter with a simultaneously prepared solution obtained by treating 10 c.c. of the standard uric acid solution (0.2 mg. of uric acid) in a 50 c.c. flask with 5 c.c. of sodium cyanide solution, 1 c.c. of the reagent, and diluting to the mark at the end of 5 minutes. The reading of the standard, divided by the reading of the unknown, multiplied by 0.2, gives the mg. of

¹ *Uric acid reagent.* 100 gm. of pure sodium tungstate (preferably Merck's or J. T. Baker's C. P.) are placed in a litre Pyrex flask and dissolved in about 600 c.c. of water. 5 gm. of pure arsenic acid (As_2O_5) are now added, followed by 25 c.c. of 85 % phosphoric acid and 20 c.c. of conc. HCl. The mixture is boiled for about 20 minutes, cooled and diluted to 1 litre. The reagent keeps indefinitely. *Sodium Cyanide.* A 5 % solution prepared fresh once in about 2 months. *Uric Acid Standard.* Stock solution. Dissolve 9 gm. pure crystallised Na_2HPO_4 and 1 gm. crystallised NaH_2PO_4 in 200–300 c.c. of hot water. The solution is filtered if not perfectly clear, made up to about 500 c.c. with hot water, and thus hot or warm (but perfectly clear) solution is poured upon exactly 210 mg. pure uric acid suspended in a few c.c. of water in a litre volumetric flask. The flask is agitated for a few minutes until the uric acid is completely dissolved. The liquid is cooled, exactly 1.4 c.c. of glacial acetic acid added, and the whole diluted to the mark and mixed. About 5 c.c. of chloroform are added to prevent growth of bacteria or moulds. The solution should keep about 2 months unless in an excessively warm room. 5 c.c. contain 1 mg. of uric acid. To make a dilute standard containing 0.2 mg. of uric acid in 10 c.c. transfer 50 c.c. of above standard to a 500 c.c. volumetric flask, dilute to about 400 c.c. with distilled water. Add 25 c.c. of dilute HCl (1 part of conc. HCl to 9 of water), dilute to 500 c.c. and mix. Prepare fresh every 2 weeks. For another procedure see *J. Biol. Chem.*, **83**, 109, 1929.

uric acid in 10 c.c. of the diluted urine. For adults on a mixed diet the average excretion of uric acid is about 0.7 gm.

Method of Folin and Wu (J. Biol. Chem., 1919, 38, 459):—Since certain other substances present in urine may produce a similar blue colour with the uric acid reagent, it is sometimes desirable to separate the uric acid from them. Separation as the silver salt is then employed. Transfer 2 to 5 c.c. of urine and about 3 c.c. of water to a centrifuge tube. Add 3 c.c. of clear acid silver lactate and centrifuge 2–3 minutes. Add a drop of silver lactate solution to the supernatant fluid. If a precipitate forms, too much urine has been used. Repeat using a smaller amount. If no precipitate forms, pour off the supernatant fluid as completely as possible. Transfer 5 c.c. of standard uric acid solution¹ containing 0.5 mg. uric acid to a 100 c.c. volumetric flask. Add to the standard from a burette 2 c.c. of 15% sodium cyanide, and add the same amount to the precipitate in the centrifuge tube. Stir the latter to complete the solution, then rinse into a 100 c.c. flask, using 20 c.c. of 20% sodium carbonate solution from a cylinder, and add 5 c.c. of water to balance that in the standard. Add 20 c.c. of sodium carbonate to the standard, and then, with shaking, 5 c.c. of the uric acid reagent¹ to each flask. Leave for 5 minutes. Then shake for a few seconds, make up to the mark with water and shake vigorously for a few seconds more. After mixing, pour out (or into test tubes) about 40 c.c. from each flask. This facilitates the settling of the precipitate, which is a decomposition product of the excess of uric acid reagent. Pour off the clear supernatant fluids into colorimeter cups and compare. The cyanide and hydrocyanic acid formed from it in acid solution are very poisonous. Pour all discarded solutions directly into drain pipes. The reading of the standard over the reading of the unknown, times 0.5 mg., gives the amount of uric acid in the amount of unknown urine taken.

¹ *Uric Acid Reagents for Folin-Wu Method.*—Standard uric acid solution.

Stock Solution.—Transfer exactly 1 gm. of uric acid to a dry funnel inserted into a volumetric flask (1,000 c.c.) and shake the uric acid into the flask as completely as possible. Dissolve 0.6 gm. of lithium carbonate in about 120 c.c. of hot water and filter. Dilute with about 60 c.c. of water and heat to 65°. Warm the volumetric flask in hot water and add hot lithium carbonate solution, rinsing the funnel and watch-glass used in weighing free from the last traces of uric acid. Shake until dissolved (usually 5–15 minutes). Cool and dilute to about 800 c.c. Add 10 c.c. clear 37–40% formaldehyde (Merck) and mix. Dilute 15 c.c. of conc. sulphuric acid with about 100 c.c. of water and cool. Add to urate and formalin mixture, dilute to volume and mix. The solution will keep for at least several months.

Standard Uric Acid Solution for Urine Method.—Dilute 10 c.c. of the stock solution to 100 c.c. with distilled water.

Uric Acid Reagent.—Transfer 100 gm. of sodium tungstate to a 2 litre flask and add 750 c.c. of distilled water. Shake until dissolved. A little white calcium precipitate may

Folin-Shaffer Method.—For larger amounts of urine or where a colorimeter is not available this is probably the best method. Phosphates and some organic matter are removed by means of uranium acetate and ammonium sulphate. The uric acid is precipitated as ammonium urate and titrated with potassium permanganate. Introduce 100 c.c. of urine (more if the sp. gr. is below 1.020) into a 350 c.c. beaker, and while stirring add from a pipette 25 c.c. of Folin-Shaffer reagent (500 grm. of ammonium sulphate and 5 grm. of uranium acetate in 710 c.c. of 0.84% acetic acid solution). Allow the mixture to settle (5–10 minutes). Filter through a dry filter paper into a dry beaker or flask. Transfer 100 c.c. of the filtrate (more if dilute) into a 300 c.c. beaker, and add, while stirring, 5 c.c. of concentrated ammonium hydroxide. Leave for 24 hours. Transfer the precipitate to an acid-washed or hardened filter paper, or better, an asbestos filter in a Hirsch funnel or Gooch crucible, and wash free from chlorides with 10% ammonium sulphate solution, using not more than 100 c.c. of this solution and using the same amount in different determinations. Transfer asbestos or uric acid from the filter paper (according to Garry: *Biochem. J.*, 1924, 18, 913, the paper may also be transferred) to the 350 c.c. beaker with the aid of 100 c.c. of hot water. After cooling add gradually, with stirring, 15 c.c. of conc. sulphuric acid. Titrate with *N*/20 potassium permanganate, keeping the mixture at 60°, to a pink end-point lasting for 10 seconds. Multiply the number of c.c. of permanganate used by 3.75 mg., by five-fourths to correct for dilution, and add 3 mg. to correct for the solubility of the ammonium urate in the amount of solution used. The result is mg. of uric acid in 100 c.c. of original urine.

For a discussion of other methods for determining uric acid in the urine see Wiechowski in Neubauer-Huppert: *Analyse des Harns*, Vol. 2, 1913.

Determination of Uric Acid in Blood.—Normal human blood usually contains from 2–3.5 mg. of uric acid per 100 c.c. In early

remain. Add 80 c.c. of 85% phosphoric acid. Close the mouth of flask with a funnel and two watch glasses, one small and one large, and boil gently but continuously for two hours. If very dark in colour, bleach with a few drops of bromine and boil for 10–15 minutes to remove excess. Cool and dilute to 1 litre.

Sodium Cyanide Solution.—A 15% sodium cyanide solution in 0.1*N* NaOH. It should be at least 1–2 days, but not a month old. Use C. P. cyanide. (Merck's is good.)

Silver Lactate Solution.—Dissolve 100 grm. of silver lactate in 700 c.c. warm water. To 100 c.c. 85% lactic acid add 100 c.c. 10% NaOH. Pour this lactic acid mixture into the silver lactate solution, dilute to 1 litre. Allow the mixture to settle and use the supernatant fluid.

interstitial nephritis values of 3-10 mg. are noted. Uric acid may increase in the blood in this condition sooner than urea or creatinine, and the determination is therefore of especial value in early nephritis. Values of 4-10 mg. are usually found in gout, and values of 2-8 mg. are found in arthritis. It is uncertain to what extent the high results in these conditions are associated with renal deficiency which often coexists. High values are found in leucaemia, owing to excessive breakdown of nuclear material. Many conditions causing renal damage raise the uric acid level. The uric acid level is reduced by administration of cinchophen, salicylates and other drugs.

Benedict's method is the simplest and gives results of clinical value. It is not applicable to animal bloods which contain material amounts of the interfering substance, thiasine, and may give high results on human blood in certain conditions. The isolation procedure of Folin and Wu is therefore more reliable.

The blood must first be deproteinised. Transfer 5 c.c. of oxalated blood (20 mg. potassium oxalate for 10 c.c.) to a 100 c.c. flask. Add 7 volumes of water (35 c.c.) and 5 c.c. of a 10% solution of sodium tungstate and mix. Add from a pipette, very slowly with constant shaking, 5 c.c. of $\frac{2}{3}N$ sulphuric acid. Close with a rubber stopper and shake. Leave for 10-20 minutes and filter on a filter that will hold everything, adding first a few c.c. of the mixture and then, when the whole filter paper has been moistened, adding the rest.

Benedict's Method.—Five c.c. of blood filtrate are transferred to a test tube of 18-20 mm. diameter, and 5 c.c. of water added. Five c.c. of standard uric acid solution containing 0.20 mg. uric acid are also made up to 10 c.c. This standard solution is made by taking 10 c.c. of the stock phosphate uric acid standard, as described under the method for urine, transferring it to a 500 c.c. volumetric flask about half full of distilled water, adding 25 c.c. of dilute HCl (1 part conc. HCl to 9 of water) and making up to 500 c.c. To both standard and unknown are added from a burette 4 c.c. of 5% sodium cyanide solution containing 2 c.c. of concentrated ammonia per litre. To each tube is then added 1 c.c. of the arsenic phosphoric tungstic acid reagent (see Urine Method). The contents of each tube should be mixed by one inversion immediately after addition of the reagent and placed at once in boiling water, where the tubes should be

left for 3 minutes after immersion of the last tube, but the time elapsing between the immersion of the first and last tubes should not exceed 1 minute. After the 3-4 minutes' heating the tubes are removed and placed in a large beaker of cold water for 3 minutes and matched in a colorimeter with the standard as soon as convenient (preferably within 5 minutes after removing from cold water). If clouding occurs (usually due to too much oxalate in the blood), the test is repeated, another 5 c.c. of water being added to the standard and unknowns just previous to heating. The reading of the standard over reading of unknown, times 4, gives mg. uric acid in 100 c.c. of blood.

Folin's Isolation Procedure.—Transfer 5 c.c. of the blood filtrate to a centrifuge tube. Add 7 c.c. of silver lactate solution (for reagents see urine method). Do not stir. Let the mixture settle for 1-2 minutes and then centrifuge. The whole of the uric acid is in the precipitate. Decant the supernatant fluid as completely as possible and add 1 c.c. of 10% NaCl in $N/10$ HCl. Stir thoroughly with a fine glass rod. Add 4 c.c. of water and stir again. Centrifuge. Pour the supernatant solution as completely as possible into a test tube graduated at 25 c.c. Transfer 5 c.c. of the standard uric acid solution (0.02 mg. of uric acid) to another similar test tube. This standard is made by half filling a 250 c.c. volumetric flask with distilled water, adding exactly 1 c.c. of standard stock solution of uric acid, 10 c.c. $\frac{2}{3}N$ sulphuric acid, and 1 c.c. of formalin diluting to volume and mixing. This standard should keep for several weeks. To each tube add 2 c.c. of the 15% cyanide solution and 2 c.c. of water. Mix well. Add 1 c.c. of uric acid reagent to each tube, dropping it directly into the solution (not down the sides). Leave for 2 minutes at room temperature, then put in a boiling water-bath for 2 minutes (not longer). Cool at once and dilute promptly to the 25 c.c. mark. Mix and compare in the colorimeter. The reading of standard over reading of unknown, times 4, gives the mg. of uric acid in 100 c.c. blood.

Urates

Uric acid is a feeble acid which possesses a dibasic function. The salts of the formula $M_2C_5H_2O_8N_4$, commonly called neutral or normal urates, dissolve readily in water, and are exclusively laboratory products, not being met with in the animal system under

either healthy or pathological conditions. The acid urates, or "di-urates," of the formula $MH(C_5H_2O_3N_4)$, are very sparingly soluble.

A third series of urates was postulated by Roberts (*Croonian Lectures*, 1892) and believed by him to be the physiological form of uric acid. These were called quadri-urates or hemi-urates and were thought to have the formula $MH(C_3H_2O_3N_4)H_2(C_5H_2O_3N_4)$.¹ For a detailed discussion of the quadri-urates see the Fourth Edition of this work. According to more recent work, however, the quadri-urates are really mixed crystals of acid urates and uric acid or solid solutions of one in the other and not substances of constant composition (See Ringer and Schmutzer: *Z. physiol. Chem.*, 1914, 89, 321).

Monosodium Urate, $C_5H_3N_4O_3 + 1\frac{1}{2}H_2O$, generally forms a crystalline powder, which, under the microscope, appears in needles (often crossed), rosettes, stellate and hedgehog-like forms (Fig. 26, page 418). It requires about 1,200 parts of cold or 120 of boiling water for solution. It may be prepared from absolutely pure uric acid by dissolving in the calculated amount of $N/10$ NaOH and at once sucking free from undissolved residue. Even in the dry form it decomposes in a few weeks. Uric acid forms two types of monobasic salts corresponding to the lactam and lactim forms of uric acid. For properties of these two types of salts see Table on page 403.

The solubility of sodium hydrogen urate in water impregnated with salt and other substances has an important bearing on the cause and cure of gout, and has been investigated by Roberts, from whose results it appears that the solvent action of the various salts depends on the nature of the metal, and has no reference to its form of combination. Salts having an alkaline reaction to litmus, like the carbonates and phosphates, behave exactly similarly to those of neutral reaction, such as the chlorides and sulphates. Salts of potassium exert no appreciable influence on the solubility of the sodium hydrogen urate in water. Salts of sodium decrease the solubility, the influence being greater the larger the proportion of salt present. Salts of ammonium, calcium, and magnesium behave similarly to, but less powerfully than, salts of sodium.

¹ Ebstein and Nicolaier (*Ueber die experimentelle Erzeugung von Harnsteinen*, Wiesbaden, 1891).

The following figures by Roberts represent the parts by weight of sodium urate dissolved at 37.8° by 1,000 parts of the solutions of the strength indicated. The amount of sodium hydrogen urate dissolved by 1,000 parts of distilled water at 37.8° was found to be 1.0.

Percentage of salt in solvent	0.1 %	0.2 %	0.3 %	0.5 %	0.7 %	1.0 %
Sodium bicarbonate.....	0.50	0.34	0.20	0.13	0.09	0.08
Sodium chloride.....	0.45	0.30	0.16	0.10	0.08	0.05
Sodium phosphate (crystallised).....	0.70	0.32
Sodium sulphate (crystallised).....	0.55	0.24
Sodium salicylate.....	0.65	0.36	0.25
Potassium hydrogen carbonate.....	0.96	1.00	1.00	0.97	1.02	0.98
Potassium chloride.....	0.96	1.01	1.10
Potassium phosphate.....	1.01	1.00
Ammonium chloride.....	0.85	0.50	0.42	0.35
Calcium chloride.....	0.27
Calcium sulphate.....	0.65	0.44
Magnesium chloride.....	0.85	0.68
Magnesium sulphate (crystallised).....	0.90

Crystalline sodium hydrogen urate is ten times as soluble in boiling water as in cold, but a saturated hot solution does not deposit the excess of salt immediately on cooling. The di-urate remains in complete solution for a considerable time, and is not entirely deposited for some days. Roberts has shown that this behaviour is not merely due to supersaturation of the liquid, but is owing to the formation of a gelatinous modification of the di-urate of greater solubility than the crystalline form. Thus, if a saturated solution of sodium hydrogen urate in boiling water is prepared, and when cold mixed with an equal volume of a 20% solution of common salt, a voluminous gelatinous precipitate will be thrown down. Saturated solutions of solid crystals of other salts (*e. g.*, sodium phosphate or acetate, potassium chloride, phosphate, acetate, etc.) may be substituted for the common salt. The precipitate, if filtered off, allowed to drain, and cautiously washed with cold water, consists of sodium hydrogen urate in a state of approximate purity. It dissolves at 37.8° in blood-serum, or in a liquid containing 0.5 grm. of sodium chloride and 0.2 of sodium carbonate per 100 c.c. (which represents the saline ingredients of serum), sufficiently freely to cause a considerable separation of uric acid after acidifying with acetic acid; whereas crystalline sodium hydrogen urate is taken up by water at 37.8° so slightly that no deposition of uric acid is obtainable on acidifying the liquid (compare p. 404).

The gelatinous form of the sodium di-urate gradually changes into the crystalline variety, and the gradual deposition of the salt from its solution in water, blood-serum, or imitation serum is evidently due to the same change of condition.

Potassium hydrogen urate, KHUr , is said to be sometimes formed as a urinary deposit in cases of fever. It is amorphous, and is more soluble than the corresponding sodium salt, requiring for solution only 800 parts of cold, or from 70 to 80 parts of boiling water.

Lithium hydrogen urate, LiHUr , forms crystalline grains, soluble in 370 parts of cold, or 39 of boiling water. Lipowitz states that if equal parts of uric acid and lithium carbonate are treated with 90 parts of water at blood-heat, a clear solution is obtained, while at 100° four times the amount of uric acid can be dissolved without increasing the weight of the lithium carbonate. Seeing that lithium carbonate itself requires about 200 parts of water for solution, its solvent action on uric acid is remarkable, and is of much interest in connection with the extensive application of lithium salts in the treatment of gout. On the other hand, it is stated by L. Siebold (*Year-book Phar.*, 1889, p. 413), as the result of direct experiment, that the relative solvent action of solutions of lithium, sodium, and potassium carbonates on a given weight of uric acid, under equal conditions of dilution and at temperature of 37° (blood-heat), is strictly proportional to the ratio of the molecular weight of these solvents. Hence lithium carbonate has the advantage that 74 parts are chemically equivalent to 106 of the sodium salt or 138 of potassium carbonate; but there the advantage ceases. Urinary sediments are similarly dissolved by these carbonates with equal facility if molecular proportions are used, and equivalent weights of lithium, sodium, and potassium citrates produce equal alkalinity in the urine of the person taking them. Siebold further states that lithium chloride and sulphate have no solvent action on uric acid and acid urates, and that natural mineral waters containing these salts have none beyond that exercised by basic constituents simultaneously present, and by the water.

Ammonium hydrogen urate, $(\text{NH}_4)\text{HUr}$, is soluble in about 1,500 parts of cold water, and quite insoluble in saturated solutions of ammonium chloride and sulphate (compare page 408). The urinary excrement of serpents is commonly stated to consist almost wholly of a mixture of ammonium hydrogen urate with free uric acid. Guano,

the excrement of various aquatic birds, consists chiefly of oxalate and hydrogen urate of ammonium in admixture with phosphates. Guanine (page 382) is also a constituent of guano, and replaces uric acid in the urine of spiders and other invertebrate animals. The aqueous solution of ammonium urate reacts acid. The ammonia is driven off by boiling with water. The salt decomposes above 60° .

Piperazine urate is described on page 213.

Neutral or normal urates of the light metals do not exist naturally, but they may be obtained by dissolving uric acid in the theoretical amount of alkali. The normal urates of lithium and ammonium are unknown. *Normal potassium urate*, $K_2C_5H_2O_3N_4$, forms small crystals having an alkaline reaction and alkaline taste. It dissolves, with partial decomposition into the acid salt, in about 36 parts of cold water, forming a liquid of soapy taste which froths strongly when shaken.

Normal sodium urate, $Na_2Ur + H_2O$, forms hard nodules which closely resemble the potassium salt, but are less soluble in water.

On passing carbon dioxide through a solution of the normal urate of potassium or sodium the corresponding hydrogen urate is precipitated. The same decomposition occurs by prolonged boiling of the solution, or by its exposure to air. Calcium urate is a frequent constituent of gouty deposits (S. Delépine, *J. Chem. Soc.*, 1887, 52, 469).

The urates of lead, copper, mercury, and silver are quite insoluble in water. Hence solutions of these metals are used for determining uric acid or for separating urates from urine.

There has been much discussion as to the relation that may exist between the disease called gout and the form in which uric acid exists in the body. Gudzent (*Z. physiol. Chem.*, 1909, 63, 455), believed that in gout the uric acid might exist to a greater extent in the blood as the less soluble lactim form, but the evidence for this view is inconclusive. On the other hand, Benedict found a combined uric acid in the red cells of certain animals. As there is evidence that purines may be metabolised differently according to whether they are given in the free state or in combination in nucleic acids, it is possible that in gout there might be a retarded metabolism of nucleotides rather than of the purines themselves. Increased amounts of combined uric acid or of nucleotides have not, however, been noted in gout. The evidence for a disturbed metabolism of

nucleins in gout is thus very slight. Another view is that in true gout we have a constitutional weakness of the kidney for the elimination of urates. There seems no doubt that in gout we do have an impaired elimination of uric acid.

The question as to the form in which uric acid exists in the urine is also a matter of dispute. All acid urines are supersaturated with uric acid in the sense that water made acid to the same degree will not dissolve the amount of uric acid found in such urines. On the other hand, all alkaline urines may dissolve more uric acid. In urine "supersaturated" with uric acid the failure of the acid to precipitate has been variously attributed to the existence of the uric acid in colloidal solution, to the protective action of other colloids present in urine, or to the existence of uric acid in the urine in combination with some other substance such as an amino acid. The latter suggestion is supported, for example, by Ascoli (*Arch. Sci. Biol.* (Italy) 1926, 9, 152), who shook urine with benzene to remove colloids and found no reduction in uric acid. Ultra-filtration of urine indicated that the uric acid was in crystalloidal form.

Urinary Deposits and Calculi

Urine is sometimes turbid as passed from the urethra, and all urine deposits a fine cloud of mucus on standing. Many specimens of urine, when allowed to cool and stand, deposit urinary salts. These sediments may appear as a purely amorphous or crystalline precipitate or as a mixture of amorphous and crystalline particles.

Urine which is turbid when actually passed from the urethra (and consequently at the temperature of the body) may owe its turbidity to the presence of suspended uric acid or urates; to the presence of earthy phosphates or carbonates (especially if the urine is that of a herbivorous animal); or to the presence of organised matters, such as mucus or pus. Deposits which require removal by surgical means from the kidneys, ureters, bladder or urethra, are best considered separately under the head of Urinary Calculi (page 420).

Urinary Sediments

Urinary deposits are rarely of a complex character, and hence very simple methods suffice to determine their nature. Examination under the microscope is especially suited for this purpose, since it is simple and readily applied, and is available for a very minute

quantity of the sediment. The urine to be examined should be allowed to stand for 12 hours in a conical glass, so that any deposit may collect at the apex, or the urine is placed in a centrifuge and the sediment collected after a few minutes of rotation. A drop of the liquid containing as much sediment as possible should then be withdrawn by a pipette, and placed on a microscope slide. It should be covered with a thin glass, and examined under an inch objective, which may subsequently be changed for a $\frac{4}{10}$ or $\frac{1}{4}$ in.

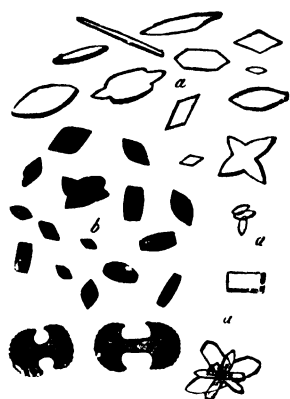


FIG. 24.—Crystals of uric acid *a*, From decomposition of urates; *b*, from human urine; *c*, dumb-bell forms.

objective. Epithelium, mucus globules, and pus cells will be distinguished as organised deposits. Urates and amorphous phosphates appear as opaque particles. Uric acid, which to the naked eye appears as a coloured sandy deposit, is distinguished under the microscope by its peculiar crystalline form and yellow or brown colour. Urine which has been allowed to stand for some time frequently contains a deposit of calcium oxalate, which is seen under the microscope as delicate octahedra. On adding a drop of sodium hydroxide on the slide, a deposit of uric acid will at once dissolve. $N/10$ hydrochloric acid, on the contrary, leaves uric acid unaffected, or causes a further deposition of minute leaflet crystals under the eye of the observer, while it at once dissolves earthy phosphates or carbonates, whether crystalline or amorphous. The organised deposits are more or less liquefied by alkali, but dilute acid leaves them unchanged. The addition of a minute amount of staining material, either finely powdered or in solution, at the bottom of the depositing vessel, will stain epithelium and other organised deposits, and thus facilitate their recognition under the microscope.

Uric acid appears under the microscope in a variety of forms. Quadratic prisms, single and in groups, spiculæ, aigrettes, and "dumb-bell" forms are common, as also are somewhat oval crystals attached together so as to form figures of eight, stars, or crosses (Fig. 24). From urine acidified with 5% by volume of hydrochloric acid, square crystals are deposited, having two opposite sides smooth and the alternate sides jagged (Fig. 25). Uric acid crystals dissolve

on adding alkali hydroxide, and are reprecipitated in minute but characteristic forms on subsequently adding hydrochloric acid.

Sodium hydrogen urate usually forms amorphous deposits, but sometimes occurs as bundles or tufts of acicular crystals, or in spheroidal masses. (Fig. 26.) Potassium and magnesium urates are almost always amorphous. **Ammonium urate** occurs only in alkaline urine, and generally in association with magnesium ammonium phosphate. It forms irregular, club-like crystals or thornapple spherules (Fig. 27). Urates are readily distinguished from phosphates by their solubility when warmed in their supernatant urine. When treated with acetic acid, deposits of phosphates dissolve, but urates change into characteristic forms of uric acid without previously undergoing solution.

Hippuric acid, according to Gorup-Benzanez, is occasionally met with in sediments from the urine of human patients who have taken benzoic acid or foods such as prunes and cranberries containing it. It occurs frequently in sediments from the urine of herbivorous animals. Hippuric acid forms characteristic acicular crystals and rhombic prisms (see Fig. 30, page 427). Some of the broader crystals resemble those of ammonium magnesium phosphate, but are insoluble in acetic acid. From uric acid they are distinguished by their solubility in alcohol. If the alcoholic solution is evaporated to dryness, and the residue dissolved in warm water, characteristic crystals of hippuric acid will be obtained on evaporation.

Magnesium ammonium phosphate, often described as "triple phosphate,"¹ is deposited from urine as soon as the liquid becomes

¹ The term "triple phosphate" should be abandoned as unscientific and misleading.

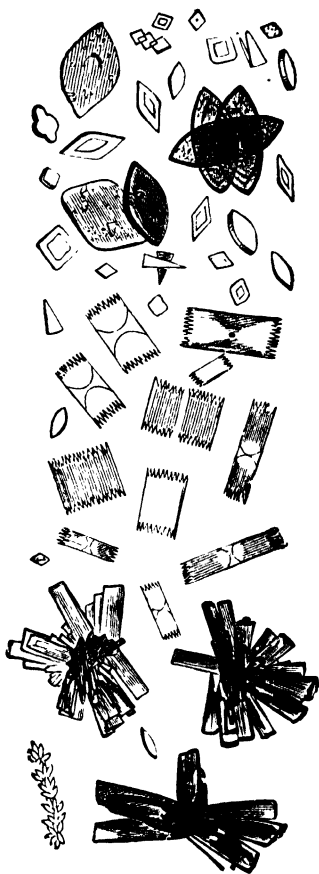


FIG. 25.—Crystals of uric acid.

alkaline from the decomposition of the urea. The natural deposit may be imitated by adding ammonium carbonate to urine, filtering the liquid immediately, and allowing the filtrate to stand for some hours. Magnesium ammonium phosphate forms fine, vitreous,

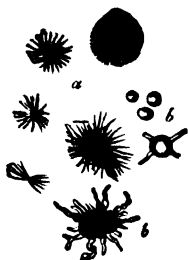


FIG. 26.—Acid urate of sodium. *a*, Needles, usually aggregated; *b*, spheroidal masses.



FIG. 27.—Ammonium hydrogen urate.

prismatic crystals ("coffin-shaped"), or ragged arborescent or stellate forms (Fig. 28).

Calcium phosphate commonly occurs as an amorphous deposit, which to the naked eye resembles pus or granular organic matter.

When precipitated from the urine by heat this deposit has been mistaken for albumin, but is distinguished therefrom by readily dissolving on adding a drop of acetic acid. The same character, and its

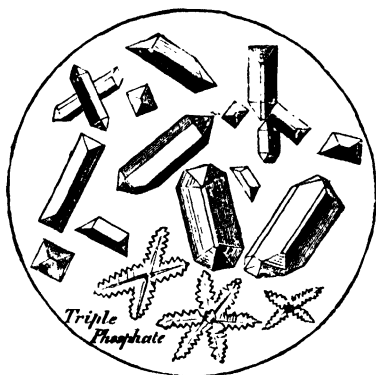


FIG. 28.—Ammonium magnesium phosphate.



FIG. 29.—Crystals of calcium oxalate.

insolubility in the supernatant urine on warming, distinguish it from deposits of amorphous urates. Under the microscope, amorphous calcium phosphate appears as minute pale granules, arranged in

irregular patches. **Magnesium phosphate** has a similar microscopical appearance and character. The crystalline form of calcium phosphate is a comparatively rare deposit. Under the microscope it appears as crystalline rods, frequently grouped in stars or rosettes, or in club or wedge-like forms, which always show the lines of crystallisation.

Calcium oxalate usually occurs in minute octahedral crystals in dumb-bell forms (Fig. 29), requiring a $\frac{1}{4}$ -in. objective for their recognition; it is liable to escape detection unless the phosphates are first dissolved by acetic acid. It occurs in certain morbid conditions, and in the urine of persons after eating rhubarb, sorrel, asparagus or spinach. The deposit may be readily induced by adding a crystal of oxalic acid to normal urine, and allowing the liquid to stand for some hours.

Calcium carbonate is rarely found as a deposit in human urine, but frequently in that of the horse and other herbivora. Under the microscope, the deposit appears as minute spherules or dumb-bells, which show a well defined black cross when viewed by polarised light. It dissolves in warm acetic acid, with effervescence, which may be observed under a low microscopic power, if a number of crystals are treated beneath a large cover-glass.

Calcium sulphate in the form of acicular crystals is said to have been observed as a urinary deposit.

Cystine occurs very rarely as a urinary deposit. It takes the form of hexagonal plates (Fig. 7, page 276), which are soluble in ammonia, and are redeposited in a more perfect form by allowing the resulting solution to evaporate spontaneously.

Leucine (page 252), **tyrosine** (page 258), **xanthine** (page 377), and **cholesterol** (Vol. II) occasionally occur as urinary deposits.

Organised deposits of various kinds are apt to occur in urine. Blood-corpuscles, epithelial cells, tube-casts, pus-corpuscles, fat globules, and spermatozoa are all more or less common under certain conditions. Their recognition depends on the employment of a microscopic power of 300 or 400 diameters with the aid of staining agents.

For the more formal chemical examination of urinary deposits, the turbid urine should be warmed to about 50° and filtered at that temperature. Deposits of earthy phosphates, calcium oxalate, uric acid, or organised matters are not dissolved on heating, and hence will be

found on the filter, but urates mostly dissolve and are re-deposited from the filtrate in a comparatively pure form on cooling. The uric acid in such deposits may be identified by the murexide test (page 404), while the resultant residue may be ignited and employed for identifying the base. Deposits of urates are often pink or red, owing to the presence of pigment, which may be removed from the deposit by treatment with alcohol.

The portion of the urinary deposit which does not dissolve on warming may be treated with *N*/10 hydrochloric acid, which will dissolve the earthy phosphates without affecting uric acid or the organised deposits. Or the insoluble portion of the deposit may be warmed with dilute acetic acid with the liquid filtered. From the acetic acid solution, calcium may be thrown down by ammonium oxalate. If the filtrate is rendered strongly alkaline with ammonia, a white crystalline precipitate, giving streaks in the track of a glass rod, consists of magnesium ammonium phosphate. If, after standing for some time, the ammoniacal liquid is filtered and treated with a drop or two of magnesia mixture, and again stirred, a further precipitation of magnesium-ammonium phosphate proves that calcium phosphate was present in the original deposit. The portion of the deposit insoluble in acetic acid may contain uric acid and calcium oxalate. The former is readily detected by the murexide test, by its insolubility in cold hydrochloric acid, and by its microscopic appearance. The calcium oxalate is insoluble in acetic acid, but dissolves in hydrochloric acid, and is reprecipitated on adding excess of ammonium acetate to the resulting solution.

Urinary Calculi

Concretions frequently occur in the bladder, kidneys, and other parts of the urinary passages. These concretions, or urinary calculi, vary greatly in form, consistence, and composition, and according to their size are known as stones, gravel, or sand. Though sometimes homogeneous, they are more frequently composed of concentric layers, and are always formed on a nucleus, which may generally be distinguished from the adjacent portions and sometimes consists of a foreign substance. Uric acid, ammonium, potassium, sodium, calcium and magnesium urates; iron, calcium, and magnesium phosphates; ammonium, calcium and magnesium carbonates (especially in calculi from herbivora); calcium oxalate; ammonium

hippurate; cystine; xanthine; ferric oxide; silica; silicates (in sheep's urine); mucus; blood; colouring and other extractive matters; have all been mentioned as the constituents of calculi. The most common kinds of human urinary calculus are: *a.* Uric acid, with urates of calcium and ammonium; *b.* magnesium ammonium phosphate, with calcium phosphate and carbonate; *c.* uric acid, with phosphates, *d.* calcium oxalate. Nearly all of these types of calculi contain some uric acid, but the percentage amount is usually low, phosphate and oxalate making up the major portion of most calculi. Calculi consisting essentially of cystin, xanthine, and other compounds, are occasionally, but very rarely, met with.

The following analyses illustrate the percentage composition of some typical kinds of urinary calculus:

Uric acid calculi	A	B
Uric acid.....	92.8	84.69
Urates.....	3.2	9.03
Ammonium magnesium phosphate.....		1.12
Extractive matters.....	1.0	2.61
Water.....	3.0	

Phosphatic calculus	Oxalate calculus
Sodium urate..... 9.77	Calcium oxalate..... 63.5
Calcium phosphate..... 34.74	Calcium phosphate..... 6.2
Ammonium magnesium phosphate..... 38.35	Water and organic matters..... 30.3
Calcium carbonate..... 3.14	
Magnesium carbonate..... 2.55	
Extractive matters..... 6.87	

J. Horbaczewski (*Z. physiol. Chem.*, 1894, **18**, 335) gives the following analyses showing the percentage composition of certain rare urinary calculi:

	Fatty concretion	Cholesterol concretion
Water.....	2.5	3.76
Ash.....	0.8	0.55
Organic matters insoluble in ether.....	11.7	0.15
Organic matters soluble in ether.....	85.0	95.84
Containing:		
Free fatty acids;.....	51.5	
Neutral fats.....	33.5	
Cholesterol.....	traces	95.87

The concentric layers of urinary calculi are frequently distinct in composition as well as in appearance, and a curious alternation of material is at times observed; uric acid, for instance, changes place with urates, phosphates, oxalates, etc. A nucleus of uric acid is generally enclosed with an external coat of phosphates,¹ but the reverse of this appears never to occur. The exterior layers in calculi of various composition are generally phosphatic. The oxalate calculi are usually the hardest, the phosphatic the softest.

Uric acid calculi are frequently met with. When composed almost wholly of uric acid, a minute portion, heated on platinum foil, chars, burns, and leaves scarcely a trace of ash. Such calculi are usually brownish-red, smooth, or tuberculated, and are composed of concentric laminæ.

Ammonium urate calculus is uncommon. It is clay-coloured, smooth and composed of fine concentric laminæ. This calculus is wholly volatile on ignition.

Cystine calculi are very rare. They are usually small, semi-transparent, smooth, of a greenish or brownish-yellow colour, and insoluble in water, alcohol, or ether. They are soluble in ammonia, and the ammoniacal solution leaves the cystine in hexagonal plates when treated with acetic acid or allowed to evaporate spontaneously.

Xanthine calculus is of very rare occurrence. It is pale brown, of a polished appearance, and soluble in alkaline liquids. On treatment with hydrochloric acid a xanthine calculus yields a solution which, on cooling, deposits xanthine hydrochloride in hexagonal scales (page 381).

Cholesterol often occurs largely in gall-stones or biliary calculi, but only rarely forms an essential part of urinary calculi. The same remark is true of bile-pigments and bile-acids.

Calcium oxalate often occurs alone, forming a deep red-brown or grey, very hard calculus, tuberculated on the exterior, and called from its appearance "*mulberry calculus*." Smaller and smooth concretions of calcium oxalate often appear as "*hemp-seed calculi*." Calcium oxalate occurs in large quantities in horse's urine, and often as concretions in pig's urine.

Calcium phosphate occasionally forms concretions of a pale colour, composed of regular laminæ.

¹ The external layers of phosphates represent a damaged condition of the urinary apparatus consequent upon the growth and presence of the uric acid or other nucleus.

Calcium sulphate calculus has been met with in only one recorded case.

Magnesium ammonium phosphate forms white, brittle, and crystalline calculi having an uneven surface. These are fairly common and are generally associated with an infection of the bladder leading to ammoniacal decomposition of the urine.

"*Fusible calculus*" is a mixture of calcium phosphate and ammonium magnesium phosphate. Such calculi are of frequent occurrence, and derive their name from the readiness with which a fragment aggregates and even fuses to a bead when heated on a platinum wire before a blowpipe. The fusibility increases with the proportion of magnesium ammonium phosphate contained in the calculus, calcium phosphate being infusible. Fusible calculi are rarely laminated. They are usually white, soft as chalk, and often are very large.

Analytical Examination of Calculi

If the calculus is entire, and sufficiently large to allow of the process, it should be sawn in half to ascertain whether it is homogeneous or built up of different concentric layers. If the latter, portions of each layer should be flaked off and examined separately.

A preliminary examination should be made by carefully applying the following tests:

1. Heat a small fragment of the sample on platinum foil, and observe the result. Cholesterol melts and burns freely. Fibrin will give an odour of burnt feathers, and cystin a smell of burning sulphur. If the calculus consists wholly of uric acid, ammonium urate, cystin, xanthine, cholesterol, or other organic matter, it will be entirely volatilised on ignition. Any residue may consist of magnesium oxide, or of potassium or sodium carbonate derived from urates previously existing; calcium carbonate originally existing as such or derived from calcium urate or oxalate; calcium or magnesium phosphate; and traces of silica, oxide of iron, etc. Any residue left after ignition should be taken up on a loop of platinum wire moistened with hydrochloric acid, and examined in a Bunsen flame for the detection of sodium, potassium, and calcium.

2. Treat a second portion of the calculus with a cold solution of alkali hydroxide. The evolution of ammonia points to the presence

of ammonium urate or magnesium ammonium phosphate in the calculus. On adding a few drops of lead acetate to the alkaline liquid and boiling, a black precipitate of lead sulphide will be formed if any cystine was originally present.

3. Treat a third small quantity of the calculus with warm dilute nitric acid. Any effervescence may be due to decomposed urate or to uric acid, but more probably to the presence of calcium carbonate in the calculus. The acid liquid should then be evaporated to dryness on the water-bath. A deep yellow residue points to the presence of xanthine, but its presence should be confirmed by the additional tests described on page 379. Uric acid and urates leave a bright red residue, which on exposure to ammoniacal vapours assumes a magnificent purple tint (page 404).

Careful application of the foregoing tests will generally give adequate information as to the general nature of the calculus, and will suffice to establish the presence or absence of most of the possible constituents. In many cases it is unnecessary to make an exhaustive analysis, but when this is required the systematic process on page 425 may be advantageously employed. It presupposes the calculus to be of the most complex nature, but the results of the preliminary examination will generally allow the procedure to be materially abridged.

Xanthine and *cystine* are occasional, but rare, constituents of urinary calculi. The latter may be detected by boiling a portion of the calculus with alkali hydroxide and lead acetate, and the former by its reaction with nitric acid (page 379). When present xanthine and cystine are precipitated with uric acid when the solution obtained by boiling the calculus with sodium hydroxide is treated with hydrochloric acid. This may be separated from uric acid by treating the precipitate with warm dilute hydrochloric acid. The filtrate, when concentrated and cooled, will deposit the xanthine hydrochloride in crystalline plates. Cystine may be precipitated from the solution as the benzoyl compound (page 278). Or the calculus, preferably previously exhausted in succession with ether, alcohol, and water, may be treated with warm ammonia. On evaporating the ammoniacal solution nearly to dryness the cystine is deposited in crystalline tables, or it may be precipitated by somewhat concentrating the ammoniacal solution and adding excess of acetic acid. Xanthine may be isolated and precipitated in the same manner. Its

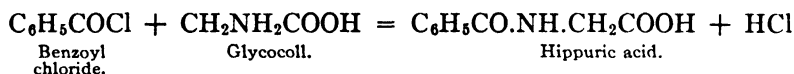
Exhaust a weighed quantity of the finely-powdered calculus (placed in a small plaited filter) with ether in a Soxhlet extractor.

Ethereal Extract is evaporated. The residue, dried at 100° and weighed, consists of <i>cholesterol</i> , <i>fatty matters</i> , and certain <i>resinous biliary matters</i> . Boil with alcohol, filter hot, and cool filtrate. <i>Cholesterol</i> will be deposited in crystals, having the characteristic angles (79° 30' and 100° 30'). To separate <i>cholesterol</i> from fatty matters, etc., saponify with alcoholic potassium hydroxide, boil off the alcohol, add water, and shake with ether, which dissolves the <i>cholesterol</i> only. The alkaline liquid acidified with dilute sulphuric acid gives <i>fatty and resinous acids</i> , which can be shaken out with ether and removed by evaporation.	Residue. Exhaust with hot alcohol, preferably without removing it from the Soxhlet extractor.	Residue. Remove from the Soxhlet-tube, boil with a moderate quantity of water, and filter hot. If the filtrate gives a deposit on cooling, the residue should be boiled again with water, but not otherwise.	Residue. Treat with warm dilute sodium hydroxide, and filter.
Aqueous Extract. Evaporate to small bulk, add excess of hydrochloric acid, and allow to stand in the cold for six hours. Filter, and wash deposit with a little cold water, and filter.	Filtrate. Divide into two equal portions. I. Distill II. Evaporate with lime, and treat distillate with stand-ard acid. The <i>ammonia</i> found existed in the calculus as <i>ammonium urate</i> .	Precipitate. Washed with alcohol, moving col-ouring matter, consists of <i>uric acid</i> existing in the calculus. It may be dried at 100° and weighed, or the uric acid may be estimated without the previous precipitation by hydrochloric acid, by the methods on <i>et seq.</i>	Precipitate. Divide into two equal portions. To one add potassium ferricyanide, if a white flocculent precipitate be formed, due to <i>proteins</i> , evaporate remaining portion of heat residue at 100° till free from acetic acid, and add water. The insoluble matter consists of <i>mucus</i> and <i>albuminous matters</i> .
Filtrate. Add excess of acetic acid, keep cold for some hours, and filter.	Filtrate. Add excess of ammonia and filter.	Filtrate. Divide into two equal parts. I. Add magnesium mixture, to re-precipitate phosphates. II. Add sodium phosphate re-maining mag-nesia, if any.	Precipitate. Add excess of ammonia, washed with ammonia water, represents the <i>ammonium phosphate</i> .
Filtrate. Wash with a little cold water and treat with warm acetic acid. Effervescence indicates <i>calcium carbonate</i> . Dilute the liquid and filter.	Filtrate. Add excess of ammonia and filter.	Filtrate. Divide into two equal parts. I. Add magnesium mixture, to re-precipitate phosphates. II. Add sodium phosphate re-maining mag-nesia, if any.	Precipitate. Add excess of ammonia, washed with ammonia water, represents the <i>ammonium phosphate</i> .
Residue. Boil with a strong solution of sodium carbonate, dilute, and filter.	Solution. Acidify with acetic acid, add CaCl_2 digest, and filter. Precipitate, washed, dried at 130°, and weighed, represents the <i>calcium oxalate</i> of the original calculus. On strong ignition it gives the CaO existing as oxalate.	Solution. Acidify with acetic acid, add CaCl_2 digest, and filter. Precipitate, washed, dried at 130°, and weighed, represents the <i>calcium oxalate</i> of the original calculus. On strong ignition it gives the CaO existing as oxalate.	Solution. Acidify with acetic acid, add CaCl_2 digest, and filter. Precipitate, washed, dried at 130°, and weighed, represents the <i>calcium oxalate</i> of the original calculus. On strong ignition it gives the CaO existing as oxalate.

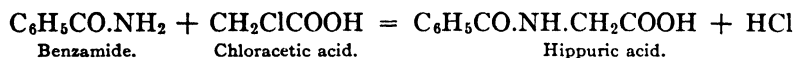
co-occurrence with cystine has not been observed, so that no separation of the two substances is necessary.

Hippuric Acid.—Benzoyl-aminoacetic acid, Benzoylglycine, $C_6H_5O_2N$; *i. e.*, $C_6H_5CO.NH.CH_2COOH$.

Hippuric acid affords a typical example of the so-called "conjugated bodies," the synthesis of which is readily effected within the living organism. Thus, if benzoic acid is taken internally, it appears in the urine as hippuric acid, and hippuric acid may be obtained artificially by heating benzoic anhydride with aminoacetic acid (glycine), or the zinc salt of the latter with benzoyl chloride:



or by the action of chloracetic acid upon benzamide:



Benzoic aldehyde, toluene, cinnamic acid, quinic acid, and phenylpropionic acid when ingested are oxidised to benzoic acid in the body and hence are also excreted as hippuric acid. Tyrosine and phenylalanine from proteins may also give rise to benzoic, and hence hippuric acid in the body, especially as these amino acids may be deaminised through bacterial action in the bowel. Substituted benzoic acids appear in the urine as substituted hippuric acids. (See Salicyluric Acid, page 429.)

The quantity of hippuric acid excreted in normal human urine averages about 0.7 grm. in 24 hours, but an increase results from a vegetable diet. This has been particularly noticed after eating plums, pears, and cranberries, and the cuticular parts of many plants act similarly. In the urine of diabetic patients, hippuric acid is frequently increased, probably owing to the increased ingestion of proteins and fruits.

It is apparently synthesized in the kidneys and elsewhere (Lewis and Griffith: *J. Biol. Chem.*, 1923, **55**, 22). Hence it is decreased in certain kidney disorders where synthetic activity of renal cells is diminished.

In the urine of herbivorous animals it is found to the extent of about 2%, its origin being doubtless in substances of the aromatic series existent in the food. Hippuric acid is also found in the urine

of other animals. Ingestion of 6-10 grm. of benzoic acid by a normal man is followed by excretion in the urine of 85-90% of it in the form of hippuric acid. The urine of birds contains instead the allied substance *ornithuric acid*, having the constitution of a dibenzoyl-diaminovaleric acid:

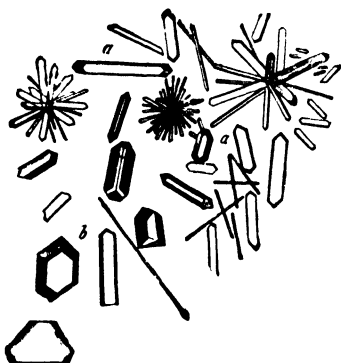
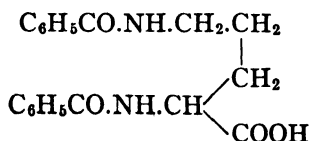


FIG. 30.—Hippuric acid (after Frey).
a, a, Prisms; b, crystals formed by slow evaporation.



FIG. 31.—Benzoic acid.

On boiling ornithuric acid with hydrochloric acid, it almost immediately parts with one benzol-group and yields benzoyl-ornithine, which on further boiling splits up into benzoic acid and diaminovaleric acid or ornithine, $(\text{NH}_2)_2\text{C}_4\text{H}_7\text{COOH}$, a base of strong alkaline reaction and of caustic taste.

When boiled for a time (half an hour) with dilute nitric, hydrochloric, or oxalic acid (or more rapidly if strong hydrochloric acid be used), hippuric acid undergoes hydrolysis, the liquid, on cooling, depositing benzoic acid, while a salt of glycocholl remains in solution $\text{C}_9\text{H}_9\text{O}_3\text{N} + \text{H}_2\text{O} = \text{C}_7\text{H}_6\text{O}_2 + \text{C}_2\text{H}_5\text{O}_2\text{N}$. This reaction is employed in practice for the preparation of glycocholl.

Hippuric acid is distinguished from benzoic and salicylic acids by its crystalline form (Figs. 30 and 31); by charring when heated with strong sulphuric acid; by giving off ammonia on ignition with soda-

lime; and by not being dissolved on agitating its solution with chloroform or petroleum spirit. When heated, benzoic acid sublimes unchanged; but hippuric acid gives red oily drops and evolves an odour of hydrocyanic acid. When precipitated by hydrochloric acid, hippuric acid separates immediately in needles, whereas benzoic acid forms scales. Hippuric acid is less soluble than benzoic acid in ether. From the neutral solution of a hippurate a neutral solution of ferric chloride throws down ferric hippurate as a cream-coloured precipitate, whereas the precipitate yielded by a benzoate with ferric chloride is reddish-brown.

Preparation

Fresh horse urine or cow urine is boiled for a few minutes with an excess of milk of lime, filtered while hot, concentrated, and the cold syrup precipitated by an excess of hydrochloric acid. The hippuric acid precipitate is collected and pressed dry on filter-paper, dissolved in milk of lime by the aid of heat, and this concentrated solution precipitated again by hydrochloric acid. The crystals are further purified by recrystallisation or by decolorisation with animal charcoal, if necessary.

Properties and Reactions

Hippuric acid crystallises in semi-transparent, long, four-sided, milk white rhombic prisms or columns or in needles by rapid crystallisation. They dissolve in 600 parts of cold water but more easily in hot water. They are easily soluble in alcohol, but with difficulty in ether. The acid dissolves more easily (about twelve times) in ethyl acetate than in ethyl ether. Petroleum spirit does not dissolve hippuric acid.

Hippuric acid melts at 187.5° to an oily liquid which crystallises on cooling. On continuing the heat it decomposes, producing a red mass and a sublimate of benzoic acid, with the generation first of a peculiar pleasant odour of hay and then an odour of hydrocyanic acid. On evaporation to dryness with nitric acid an intense odour of nitro-benzene is obtained when the residue is heated with sand in a glass tube (Lücke's reaction). For the detection of hippuric acid, K. Spiro (*Z. physiol. Chem.*, 1899, 28, 174) recommends warming the hippuric acid with acetic anhydride, anhydrous sodium acetate and benzaldehyde. After warming for $\frac{1}{2}$ hour the lactimide of phenyl-aminocinnamic acid crystallises out and has a melting-point of 165 to

166°. On heating the lactimide with strong sodium hydroxide until ammonia is given off and acidifying, phenylpyrroacemic acid, $C_6H_5CH_2CO.COOH$, separates out. This acid is soluble in ether.

Estimation

Hippuric acid may be determined by the methods of Kingsbury and Swanson (*J. Biol. Chem.*, 1921, **68**, 13), Quick: (*ibid.*, 1926, **67**, 477), or of Griffith (*ibid.*, 1926, **69**, 197).

Griffith's Method.—Place 10 c.c. of protein-free urine (containing not more than 150 mg. of hippuric acid) and 0.1 c.c. of conc. HCl in the extraction tube and 100 c.c. of ether in the 500 c.c. Kjeldahl flask. (The arrangement of the extraction apparatus is shown in Fig. 32.) Immerse the lower portion of the flask in a water-bath heated to 60–70°, and continue the extraction for one hour. Distill off the ether. To the dry residue in the flask add 5 c.c. of sodium hypobromite solution (prepared by mixing equal volumes of 25% NaOH and a solution containing 12.5 gm. of bromine and 12.5 gm. of sodium bromide dissolved in 100 c.c. of water) and shake for one minute. Add 2 c.c. of sulphuric acid (diluted 1:5). Mix thoroughly. Add 2 c.c. of 25% NaOH and 2 c.c. of the hypobromite solution and shake for one minute. The hypobromite destroys traces of urea which may be present. Proceed with the determination of nitrogen in the flask by the Kjeldahl method. If (y) represents the number of c.c. of 0.1*N* acid neutralised by ammonia, then

$$y \times 1.4 \times \frac{179}{14} = \text{mg. of hippuric acid in 10 c.c. of urine.}$$

Other methods are based upon hydrolysis of the hippuric acid and estimation of resulting benzoic acid (see references above and Vol. III of this work).

Salicyluric acid, $C_6H_5(OH)O_3N$, has the constitution of a hydroxy-hippuric acid. It occurs in the urine after administration of salicylic

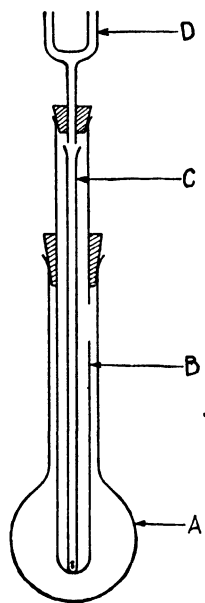


FIG. 32.—Continuous extraction apparatus. (Griffith.)

(A), 500 c.c. Kjeldahl flask. (B), glass tube (420 × 15 mm.) with side opening. (C), glass tube (400 × 6 mm.) with widened top and small openings at bottom. (D), condenser.

acid, which has the constitution of ortho-hydroxybenzoic acid, and may be detected therein by the bluish-violet coloration produced on adding dilute ferric chloride. Salicyluric acid is more soluble than hippuric acid. On boiling with hydrochloric acid, it is split up into salicylic acid and glycocholic acid.

Glycuronic acid (also *glucuronic acid*), $C_6H_{10}O_7$; *i. e.*, $COH(CHOH)_4COOH$. Glycuronic acid apparently has its origin in the dextrose of the body, to which compound it is closely related.¹ Levene and co-workers have shown it to be a component of chondroitin and mucoitin sulphuric acids (Levene: *Hexosamines and Mucoproteins*).

It was first obtained in the conjugated form of campho-glycuronic acid in the urine of dogs to which camphor had been administered, and subsequently as uro-chlorallic acid after the administration of chloral. Glycuronic acid is remarkable for its tendency to form ethereal or glucosidal compounds when appropriate substances are introduced into the body. It may also form ester linkages with substances such as benzoic acid. Traces of such compounds occur normally in urine, especially indoxyl- and skatoxyl-glycuronic acids. A combination with urea, having probably the constitution of uro-glycuronic acid, may also be present in urine.

Baeyer (*Annalen*, 1870, 155, 257) has shown that euxanthic acid, which exists in combination with magnesia in the "purée" or "Indian yellow" of commerce,² is decomposed on boiling with hydrochloric

¹ The relation between glycuronic acid and substances of the sugar-group is shown by the following constitutional formulæ:

Dextrose.....	$CH_2(OH).(CH.OH)_4.CO.H$
Gluconic acid.....	$CH_2(OH).(CH.OH)_4.CO.OH$
Saccharic acid.....	$CO(OH).(CH.OH)_4.CO.OH$
Glycuronic acid.....	$CO(OH).(CH.OH)_4.CO.H$
Gulonic acid.....	$CO(OH).(CH.OH)_4.CH_2.OH$
Gulose.....	$CO.H).(CH.OH)_4.CH_2.OH$

² Piuri or Purée, now used as a pigment under the name of "Indian yellow," is obtained in Bengal, from the urine of cows which are fed exclusively on the leaves of the mango tree and water. The urine is heated, and the precipitate separated and dried. Analyses of very pure specimens of purée by C. Graebe (*Annalen*, 254, 265) showed: euxanthic acid, 51; silica and alumina, 1.5; magnesia, 4.2; lime, 3.4; and water and volatile substances, 39%. The analyses of Stenhouse and Erdmann show much less lime. Urea, uric acid, and hippuric acid have also been found in purée. The poorer qualities contain considerable quantities of euxanthone, partly free and partly in combination. For the isolation of the euxanthic acid and euxanthone, and the assay of purée, the colouring matter should be triturated with dilute hydrochloric acid until the whole has assumed the bright yellow colour of euxanthic acid. The residue is then well washed with cold water to remove the salts, and the euxanthic acid extracted from the residue by ammonium carbonate solution. It is precipitated from the filtrate by hydrochloric acid, and purified by crystallisation from alcohol. The euxanthone, left undissolved by the ammonium carbonate, is treated with sodium hydroxide, the solution precipitated with an acid and the precipitated euxanthone shaken out with ether, or filtered off and dried at 100°.

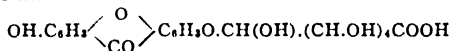
acid or dilute sulphuric acid, with formation of euxanthone and an acid which has been shown by Spiegel (*Ber.*, 1882, **15**, 1965), to be identical with glycuronic acid, $C_{19}H_{18}O_{11} = C_{13}H_8O_4 + C_6H_{10}O_7$. In fact purr   may be used for the preparation of glycuronic acid (see Fourth Edition of this work).

Preparation of Glycuronic Acid (Method of Quick: *J. Biol. Chem.*, 1927, **74**, 331). Give 5 grm. of pulverised borneol daily to each of several dogs. Collect the urine, acidify with acetic acid and add lead acetate. Most of the colouring matter is carried down. Filter, heat the filtrate to boiling, and add an excess of zinc acetate. Filter off the precipitate and wash with hot water until no more colouring matter is extracted. This is practically pure zinc borneol glycuronic acid. About one grm. is obtained for each grm. of borneol given.

Dissolve the finely powdered zinc salt in hot 3.5*N* sulphuric acid, using about 140 c.c. for each 100 grm. of the salt. When completely dissolved cool rapidly and put in an ice-box for several hours. Filter off the crystals of borneol glycuronic acid, wash with a little cold water, and dry in the air.

Dissolve 100 grm. of borneol glycuronic acid in 1,500 c.c. of 0.2*N*-sulphuric acid and boil for 3 hours beneath a reflux condenser. Filter and treat the hot filtrate with sufficient barium hydroxide to precipitate the last traces of sulphuric acid. Leave the mixture to settle. Siphon off the supernatant fluid, and complete the separation by centrifuging. Concentrate the solution under diminished pressure to a syrupy consistence, and let it stand to crystallise. Filter off the crystals and wash them with a small amount of alcohol to remove the pigment. This is a mixture of glycuronic acid and its lactone. Treat 4 grm. of the product with 200 c.c. of 95% alcohol and set aside for 12 hours. Repeat twice, using 100 c.c. portions of alcohol. The residue should be glycuronic acid of 99% purity. To obtain the pure lactone dissolve some of the mixture of acid and lactone in hot glacial acetic acid, allow the solution to cool, and recrystallise from hot water.

Euxanthic acid has the constitution:



It forms pale yellow needles, m. p. 156 to 158°. It has a sweet taste and bitter after-taste, is but slightly soluble in cold water, very sparingly in ether, but readily in boiling alcohol. Alkalies colour the solution deep yellow. Euxanthic acid does not reduce Fehling's solution, nor form a compound with phenyl-hydrazine.

Euxanthone is a neutral substance, crystallising in pale yellow needles, soluble in alkalies, but not in dilute acids. It forms no compound with phenylhydrazine.

Glycuronic acid is a syrupy liquid, readily soluble in water and slightly soluble in alcohol. When the aqueous solution is boiled, evaporated, or even allowed to stand at the ordinary temperature, the acid loses the elements of water and yields the anhydride or lactone. It is a strong organic acid ($K < 1 \times 10^{-3}$).

Glycuronic anhydride, $C_6H_8O_6$, forms monoclinic tables or needles, having a sweet taste, and m. p. 160° when heat is gradually applied, or 170 to 180° when heated rapidly. The anhydride is insoluble in alcohol, but dissolves readily in water to form a dextrorotatory solution, $[\alpha]_D = 19.25^\circ$. The solution prevents the precipitation of cupric solutions by alkalies, and powerfully reduces Fehling's solution, the copper-reducing power being 98.8, compared with glucose as 100.

Glycuronic acid itself is dextrorotatory ($[\alpha]_D = +36^\circ$), but many of its compounds are lævorotatory.¹ It shows mutarotation with an initial value of $[\alpha]_D^{20} = +16$. It reduces Fehling's solution on heating, and precipitates the metals from hot alkaline solutions of silver, mercury, and bismuth.

By treatment with bromine or nitric acid it yields saccharic acid, $C_6H_{10}O_8$, a reaction which indicates the presence of an aldehyde group and the close relation between glycuronic acid and dextrose. Saccharic acid can again be reduced to glycuronic acid by treatment with sodium amalgam, further treatment yielding gulonic acid, $C_6H_{12}O_7$, a body which does not reduce Fehling's solution. (Fischer and Piloty, *Ber.*, 1891, 24, 521). On the reduction of glycuronic acid with sodium amalgam Thierfelder (*Z. physiol. Chem.*, 1891, 15, 71) obtained *d*-gulose.

When boiled with alkali hydroxide, glycuronic acid yields oxalic acid as an invariable product. Catechol and protocatechuic acid are also formed if concentrated alkali is employed for the treatment.

Glycuronic acid is distinguished from dextrose by not undergoing alcoholic fermentation when treated with yeast, but by the action of putrefactive bacteria it splits into *l*-xylose and carbon dioxide. Some workers find small amounts to be destroyed in yeast fermentation. This relation of glycuronic acid to the pentoses accounts for the colour reactions which it gives with the furfural reagents.

¹ After taking chloral hydrate the urine contains trichlorethyl-glycuronic acid (''uro-chloralic acid''), a lævorotatory body which is decomposed into trichlorethyl alcohol and dextrorotatory glycuronic acid.

When heated with concentrated hydrochloric acid in the presence of orcinol or phloroglucinol glycuronic acid or its conjugated derivatives give the same coloration as the pentoses; namely, with orcinol, a reddish-blue colour which turns to bluish-green and which gives absorption bands between C and D and which, when shaken out with amyl alcohol, gives a bluish-green solution which has the same absorption. With phloroglucinol a cherry-red colour is obtained, and on shaking this out with amyl alcohol a red solution having absorption bands between D and E is obtained.

Glycuronic acid forms a potassium salt which crystallises in needles. The sodium salt is similar. The zinc, cadmium, copper, silver, and calcium salts are uncrystallisable. The barium salt is amorphous and soluble in water. It is the compound employed for the isolation of glycuronic acid from urine.

With phenylhydrazine, glycuronic acid forms a brownish crystalline compound melting at 114 to 115°, but under modified conditions an amorphous, brownish-yellow substance, melting at 150°, is produced. Glycuronic anhydride gives only brown globules with phenylhydrazine. On treating 1 molecule of glycuronic acid with 3 molecules of phenylhydrazine for 12 to 24 hours in the thermostat at 40° a glycuronic osazone, m. p. 200 to 205°, is obtained. With *p*-bromphenylhydrazine hydrochloride and sodium acetate glycuronic acid gives a *p*-bromphenyl hydrazone, which is characterised by insolubility in absolute alcohol and by its strong l  vororototy action, and which is well suited for the detection of glycuronic acid. When dissolved in a mixture of alcohol and pyridine (0.2 grm. substance in 4 c.c. pyridine and 6 c.c. alcohol) it has a rotation of 7° 25' which corresponds to $[\alpha]_D^{20} = -369^\circ$.

For properties of a number of glycuronic acid derivatives as well as of a considerable number of conjugated glycuronates see Abderhalden, *Handbuch Biologische Arbeitsmethoden*, Abt. 1, Teil 5, 1922.

On distillation with hydrochloric acid, glycuronic acid is decomposed with formation of furfuraldehyde, carbon dioxide, and water. Glycuronic anhydride and urochloralic acid undergo a similar decomposition, and a trace of furfural is also obtainable by similarly treating normal urine.

Tollens and his collaborators have proposed this reaction for the estimation of glycuronic acid and its derivatives determining either

the furfural (*Z. physiol. Chem.*, 1909, **61**, 95 and 1910 **64**, 38) or the carbon dioxide (*Ber*, 1907, **40**, 4513). The carbon dioxide obtained on distilling glycuronic acid with hydrochloric acid amounted to 26.5%, whereas the yield from dextrose or lævulose was not more than 1%. The furfural yielded by glycuronic anhydride under the same treatment was 15.23% of the weight taken. Those natural compounds which readily yield glycuronic acid on treatment with dilute acids give furfural on distillation with hydrochloric acid, and the proportion obtained is a measure of the glycuronic acid which may be separated from such compounds. Thus euxanthic acid yielded 6.16 to 7.17% of furfural; urochloralic acid, 9.88 to 10.30%; and potassium urobtychloralate, 9.50%.

Normal urine contains 0.004 gm. glycuronic acid in 100 c.c. This is chiefly conjugated with phenol, but a small quantity is combined with indole and skatole.

Quantitative Determination of Glycuronic Acid in the Urine.—

Glycuronic acid exists in the urine chiefly in conjugated forms. A satisfactory method which is applicable to all conjugated glycuronic acids is not available. Quick has described a method for menthol glycuronic acid which he claims can be extended with slight modifications to the determination of other conjugated glycuronates (*J. Biol. Chem.*, 1924, **61**, 667).

10 c.c. of filtered urine (5 c.c. if the concentration of menthol glycuronic acid is known to be high) is transferred to the extraction tube of a continuous extractor (see Fig. 429) and acidified with 1 c.c. of 20 per cent sulphuric acid. 50 c.c. of ether are put in the boiling flask and the urine extracted for 2½ hours. The etheral extract is transferred quantitatively to a 100 c.c. Erlenmeyer flask, the ether boiled off, and the residue hydrolysed by heating it for 15 minutes with 10 c.c. of *N* HCl beneath a reflux condenser. The liquid is cooled neutralised to litmus with *N* NaOH, and diluted to 25 c.c. If the concentration of glycuronic acid is 0.1% or higher, the Benedict method is used; if lower, the Folin-Wu method. For the Benedict method the solution, diluted to a concentration of 0.1 to 0.15%, and 10 c.c. of Benedict's reagent and 2 gm. of anhydrous sodium carbonate (weighed to within 0.05 gm.) are placed in a 100 c.c. Erlenmeyer flask, heated to boiling, and kept at the boiling point while the glycuronic acid solution is run in slowly until the last blue or green tint disappears. 1 c.c. of Benedict's

solution equals from 2.23–2.42 mg. of glycuronic acid as the number of c.c. added by titration varies from 14–22 c.c. The Folin-Wu method is carried out in the usual way. For details see Hawk and Bergeim: *Practical Physiological Chemistry*, 9th Edition, or use one of the sugar methods given in Volume I. Glycuronic acid has the same reducing power as glucose.

Glycuronic acid occurs in the urine to a very notable extent after the administration of morphine, chloroform, butylchloral, nitrobenzene, camphor, curare, and certain other drugs. It was undoubtedly mistaken for dextrose by the older observers.

Ketonic Derivatives

These include β -hydroxybutyric acid, acetoacetic acid and acetone, and these substances occur in the urine especially in diabetes mellitus, but also in many other diseases.

Acetoacetic acid is a normal product of the oxidation of the higher fatty acids in the body. Under ordinary conditions it is almost completely oxidised to carbon dioxide and water, but where such oxidation is interfered with, as in diabetes, the acetoacetic acid accumulates in the blood and is excreted in the urine. As carbohydrate is essential for the proper oxidation of the fats, acetoacetic acid is also found in abnormal amounts on a carbohydrate-free diet or during fasting. β -Hydroxybutyric acid is formed in the body by the reduction of acetoacetic acid. Acetone is formed from acetoacetic acid, which is unstable and readily loses carbon dioxide to form the ketone.

Normal adults on a mixed diet excrete, on the average, 3–15 mg. of combined acetone and diacetic acid (usually about one fourth as acetone) and up to 20–30 mg. of β -hydroxybutyric acid per day. In diabetic acidosis these values may increase to 5 and 50 grm., respectively, or even more. In such conditions the alkali of the blood is markedly depleted. For a discussion of acetone body formation see Shaffer: *Physiological Reviews*, 1923, 3, 394. Certain amino acids from protein may also give rise to acetone formation in the body.

β -Hydroxybutyric acid, $\text{CH}_3\text{CH}(\text{OH})\text{CH}_2\text{COOH}$, generally forms an odourless syrup, but can also be obtained as crystals. It may be isolated as the calcium-zinc salt (Shaffer and Marriott: *J. Biol. Chem.*,

16, 265, 1913. It is readily soluble in water, alcohol, and ether. It is lævorotatory, with a specific rotation of $[\alpha]_D = -24.12^\circ$ for solutions containing 1 to 11%. β -Hydroxybutyric acid is not precipitated by basic lead acetate or by ammoniacal basic lead acetate, nor does it ferment. On boiling with water, especially in the presence of a mineral acid it decomposes into α -crotonic acid which has a melting-point of 71 to 72° , $\text{CH}_3\text{CH}(\text{OH})\text{CH}_2\text{COOH} = \text{H}_2\text{O} + \text{CH}_3\text{CH}:\text{CH}.\text{COOH}$. On oxidation with a chromic acid mixture β -hydroxybutyric acid yields acetone.

Detection of β -Hydroxybutyric acid in the Urine.—If a urine is still lævogyrate after fermentation with yeast, the presence of β -hydroxybutyric acid is probable. Black gives the following reaction:—Evaporate 15 c.c. of urine to 5 c.c. on the water-bath. This removes acetoacetic acid. Add 2 drops of conc. HCl and the smallest quantity of plaster of Paris necessary to form a solid mass. Allow the mixture to set. Pulverise and extract twice with ether. Evaporate the ether, add 5 c.c. of water and a small amount of barium carbonate. To the almost neutral aqueous layer now add 2 or 3 drops of hydrogen peroxide solution, avoiding excess, shake and add a few drops of 5% ferric chloride solution containing 0.4% of ferrous chloride. Set aside for a few minutes. A red colour, due to the formation of acetoacetic acid, is noted.

Acetoacetic acid, diacetic acid, $\text{CH}_3\text{CO}.\text{CH}_2\text{COOH}$, is a colourless liquid which mixes in all proportions with water, alcohol, and ether. On heating to boiling with water, especially in the presence of acids, it splits into carbon dioxide and acetone; therefore it gives the acetone reactions given below. It differs from acetone by giving the following reactions:

Gerhardt's Reaction.—Treat 10 to 15 c.c. of the urine with ferric chloride solution until it fails to give a precipitate, filter, and add more ferric chloride. In the presence of acetoacetic acid a wine-red colour is obtained. The colour becomes paler at the room temperature within 24 hours, but more quickly on boiling (differing from salicylic acid, phenol, thiocyanates). A portion of the urine slightly acidified and boiled does not give this reaction, on account of the decomposition of the acetoacetic acid.

Arnold and Lipliawsky's Reaction.—6 c.c. of a solution containing 1 grm. of *p*-aminoacetophenone and 2 c.c. of concentrated hydrochloric acid in 100 c.c. of water are mixed with 3 c.c. of a 1% potassium

nitrite solution and then treated with an equal volume of urine. A few drops of concentrated ammonia are now added and the tube violently shaken. A brick-red coloration is obtained. 10 drops to 2 c.c. of this mixture (according to the quantity of acetoacetic acid in the urine) are then taken. 15 to 20 c.c. of HCl of sp. gr. 1.19, 3 c.c. of chloroform, and 2 to 4 drops of ferric chloride solution are added and the whole mixed without shaking. In the presence of acetoacetic acid the chloroform is coloured violet or blue (otherwise only yellowish or faintly red). This reaction is more delicate than the preceding test and indicates 0.04 grm. of acetoacetic acid. Large amounts of acetone (but not the quantity occurring in urines) give this reaction, according to Allard.

Le Nobel's Reaction.—Make 10 c.c. of urine acid with acetic acid, add a few drops of a dilute aqueous solution of sodium nitroprusside and stratify concentrated ammonium hydroxide upon the mixture. In the presence of acetoacetic acid a violet ring forms at once. Acetone also responds, but the test is more delicate for acetoacetic acid and the response is more prompt.

Bondi and Schwarz's Reactions.—Titrate 5 c.c. of the urine drop by drop with iodine and potassium iodide solution until the colour is orange-red. Then warm gently, and when the orange-red colour has disappeared, add the iodine solution again until the colour remains permanent on warming. Then boil, when the irritating vapours of iodoacetone will attack the eyes. Acetone does not give this reaction.

Acetone, CH_3COCH_3 , which occurs to a slight extent in normal urine, but in much greater amounts in diabetes mellitus and many other diseases, gives a peculiar odour to this fluid. It also gives an odour to the expired air similar to apples. Acetone boils at 56.3° , mixes with water, alcohol, and ether, and may be detected by the following reactions (see also Vol. I).

Lieber's Iodoform Test.—When an aqueous solution of acetone is treated with alkali and then with some iodo-potassium-iodide solution and gently warmed, a yellow precipitate of iodoform is formed, which is known by its odour and by the appearance of the crystals (six-sided plates or stars) under the microscope. This reaction is very delicate, but it is not exclusively characteristic of acetone. Urine is best acidified with acetic acid and about one-fourth the volume distilled over, the distillate being used for tests. Gunning's modifica-

tion of the iodoform test consists in using an alcoholic solution of iodine and ammonia instead of the iodine dissolved in potassium iodide and alkali hydroxide. In this case, besides iodoform, a black precipitate of iodide of nitrogen is formed, but this gradually disappears on standing, leaving the iodoform visible. This modification has the advantage that it does not give any iodoform with alcohol or aldehyde. On the other hand, it is not quite so delicate, but still it detects 0.01 mg. of acetone in 1 c.c.

Reynold's mercuric oxide test is based on the power of acetone to dissolve freshly precipitated mercuric oxide. A mercuric chloride solution is precipitated by alcoholic potassium hydroxide. To this add the liquid to be tested, shake well, and filter. In the presence of acetone the filtrate contains mercury, which may be detected by ammonium sulphide. This test has about the same delicacy as Gunning's test. Aldehydes also dissolve appreciable quantities of mercuric oxide.

Legal's Sodium Nitroprusside Test.—If an acetone solution is treated with a few drops of a freshly prepared sodium nitroprusside solution and then with potassium hydroxide or sodium hydroxide solution, the liquid is coloured ruby-red. Creatinine gives the same colour; but if the mixture is acidified with acetic acid, the colour becomes carmine or purplish red in the presence of acetone, but yellow and then gradually green and blue in the presence of creatinine. With this test paracresol responds with a reddish-yellow colour, which becomes light pink when acidified with acetic acid and cannot be mistaken for acetone. Rothera (*J. Physiol.*, 1908, 37, 49) has suggested a modification which is more delicate by using ammonium salts and ammonia. The chemistry of Legal's test has been studied by Cambi: *Atti. Accad. Lincei* 1913, 22, I, 376. He claims that the colour reaction is due either to: (I) the formation of a complex of ferropentacyanide with the isonitroso compound of the ketone, or to (II) the formation of such an ion with the isonitramine derivative of the ketone.

The quantity of β -hydroxybutyric acid can be estimated by completely extracting the acid by ether and determining the specific rotation. The following methods are those usually employed.

Shaffer's Method.—25 to 250 c.c., depending upon the amount of β -hydroxybutyric acid present, of the urine are placed in a 500 c.c. flask, diluted to about 300 c.c., and precipitated by an excess of basic

lead acetate (a volume equal to that of the urine, or about half that if no sugar is present) and about half as much ammonia as lead acetate. The mixture is diluted to 500 c.c., shaken and filtered. 200 c.c. of the filtrate are placed in an 800 c.c. Kjeldahl digestion flask, 300 to 400 c.c. of water, 15 c.c. concentrated sulphuric acid and a little talcum added, and the mixture distilled until 200 to 250 c.c. of the distillate have been collected. This distillate (A) contains the acetone (both preformed and that produced from the acetoacetic acid) and volatile fatty acids. It is treated with 5 c.c. of 10% potassium hydroxide solution and distilled again to remove the volatile fatty acids. This second distillate (A₂) is now titrated with standard iodine and thio-sulphate. The residue obtained from distillate (A) is then distilled with 400 to 600 c.c. of a 0.1 to 0.5% potassium dichromate solution,¹ which is added by means of a dropping tube, during the process of the distillation. When about 500 c.c. of the distillate (B) have collected add 25 c.c. of a 3% solution of hydrogen peroxide and 10 c.c. of 10% NaOH, and redistill, heating cautiously until peroxide is decomposed. Collect about 300 c.c. distillate (B₂) and titrate with iodine and thiosulphate. 1 c.c. of *N*/10 iodine is equivalent to 0.967 mg. of acetone or 1.736 mg. of β -hydroxybutyric acid. Shaffer recommends the use of solutions of thiosulphate and iodine which are a trifle stronger than *N*/10—*i. e.*, 1.034 *N*/10. Each c.c. of an iodine solution of this strength is equivalent to 1 mg. of acetone or to 1.793 mgrm. of β -hydroxybutyric acid. The titration of distillate (A₂) gives the amount of acetone (either pre-formed or produced from acetoacetic acid), and the titration of distillate (B₂) gives the amount of acetone derived from the β -hydroxybutyric acid by oxidation. The titration is carried out by adding an excess of the iodine solution and of NaOH (15–25 c.c. of 20%). Shake for about a minute. Add HCl equivalent to the NaOH added, and titrate with thiosulphate to a light yellow colour. Add starch and complete the titration. About 10% should be added to the results for β -hydroxybutyric acid as obtained by this method, as the yield of acetone is only 90% of the theoretical. The error appears to be practically constant, so that satisfactory results may be obtained by correction. Marriott has suggested a modification for blood analysis (*J. Biol. Chem.*, 1913, 16, 293). Normal

¹ In the presence of large amounts of sugar or when large volumes of the urine are used it may be necessary to use more dichromate.

blood contains only traces of acetone bodies. In diabetic coma values of 50 mg. per 100 c.c. have been obtained.

The *extraction* method suggested by Black (*J. Biol. Chem.*, 5, 207), is also very satisfactory for the quantitative determination of β -hydroxybutyric acid. The process is as follows: Take 50 c.c. of the urine under examination and make faintly alkaline with sodium carbonate; evaporate to one-third of the original volume. Further concentrate to about 10 c.c. on a water-bath, cool the residue, acidify it with a few drops of concentrated hydrochloric acid, and add plaster of Paris to form a thick paste. Permit the mixture to stand until it begins to "set," then break it up with a stout glass rod having a blunt end, and reduce the material to the consistence of a fairly dry coarse meal. Transfer the meal to a Soxhlet apparatus, and extract with ether for 2 hours. At the end of this period evaporate the ethereal extract, either spontaneously or in an air current. Dissolve the residue in water, add a little bone-black if necessary, filter until a clear solution is obtained, and make up the filtrate to a known volume (25 c.c. or less) with water. The amount of β -hydroxybutyric acid should then be determined by means of the polarimeter. Magnus-Levy (*Ergeb. d. inneren Med. u. Kinderheil.*, 1908) extracts the β -hydroxybutyric acid from the urine (100 to 1,000 c.c. according to the amount of acid present) after adding ammonium sulphate and sulphuric acid, in a Zemannowitz extraction apparatus, and evaporates the ethereal extract spontaneously to dryness. The residue is completely extracted with water, and the quantity of β -hydroxybutyric acid determined by the polariscope after filtering.

Folin-Hart Method for Acetone and Acetoacetic Acid.—Introduce into a wide-mouthed bottle 200 c.c. of water, an accurately measured excess of $N/10$ iodine solution, and an excess of 40% potassium hydroxide. Prepare an aeration cylinder, containing alkaline hypoiodite solution, to absorb any acetone which may be present in the air of the laboratory, and suspend between this cylinder and the bottle (above referred to) a test-tube about 2 in. in diameter. This large test-tube should contain 20 c.c. of the urine under examination, 10 drops of a 10% solution of phosphoric acid, 10 gm. of sodium chloride, and a little petroleum spirit, and should be raised sufficiently high to facilitate the easy application of heat to its bottom portion. The connections on the side of the tube should be provided with

bulb-tubes containing cotton. When the apparatus is arranged as described, it should be connected with a Chapman pump and an air current passed through for 25 minutes. During this period the contents of the test-tube are heated just to the boiling point, and after an interval of 5 minutes again heated in the same manner. By this means the diacetic acid is converted into acetone, and at the end of the 25-minutes period this acetone, as well as the pre-formed acetone, will have been removed from the urine to the absorption bottle, and there retained as iodoform. The contents of the absorption bottle should now be acidified with concentrated HCl and titrated with *N*/10 thiosulphate and starch.

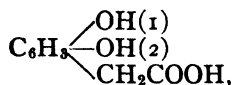
Acetone may be determined separately by first running the current of air for 20–25 minutes before applying heat. Acetone is carried over. The receiver is replaced with fresh hypoiodite solution and the process continued as above for acetoacetic acid.

The methods of Van Slyke (*J. Biol. Chem.* 1917, 32, 455) for the determination of acetone bodies based on precipitation of acetone as a basic mercuric sulphate compound are very good. Scott-Wilson precipitates with basic mercuric cyanide (*J. Physiol.*, 1911, 42, 444).

Aromatic Hydroxy Acids.—In the putrefaction of proteins in the intestine *p*-hydroxyphenyl-acetic acid, $\text{C}_6\text{H}_4(\text{OH})\text{CH}_2\text{COOH}$, and *p*-hydroxyphenyl-propionic acid, $\text{C}_6\text{H}_4(\text{OH})\text{CH}_2\text{CH}_2\text{COOH}$, are formed from tyrosine, and these pass into the urine. The quantity is increased under the same conditions as the phenols. Both of these acids are soluble in water and ether and give a beautiful red coloration on being warmed with Millon's reagent. The first one melts at 148° and the second at 125°.

To detect the presence of these hydroxy acids proceed as follows: Warm the urine for a while on the water-bath with hydrochloric acid in order to drive off the volatile phenols. After cooling, shake three times with ether, and then shake the ethereal extracts with dilute sodium hydroxide solution, which dissolves the hydroxy acids, while the phenols remain in the ether. The alkaline solution of the hydroxy acids is now faintly acidified with sulphuric acid, shaken again with ether, and the ether removed and allowed to evaporate. The residue is dissolved in water and the solution tested with Millon's reagent. The two hydroxy acids are best differentiated by their melting-points.

Besides these two hydroxy acids, we sometimes have other hydroxy acids in urines; namely, homogentisic acid, uroleucic acid, cydroxy-mandelic acid, hydroxyparacoumaric acid, and cynurenic acid (only in dog's urine). *Homogentisic acid*, dihydroxyphenylacetic acid,



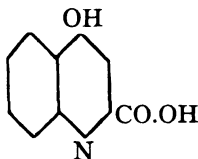
was discovered by Marshall and isolated from the urine by Wolkow and Baumann in a case of *alcaptonuria*. The formation of homogentisic acid is due to an anomaly in the metabolism of tyrosine and phenylalanine in the body of alcaptonurics, a so called *inborn error of metabolism*, the demolition of these bodies stopping at this point. Abderhalden was able to produce experimental alcaptonuria by feeding a normal man with 50 grm. of tyrosine.

Homogentisic acid crystallises with 1 H₂O in large, transparent prismatic crystals, which become non-transparent at the temperature of the room, with the loss of water of crystallisation. They melt at 146.5 to 147°. They are soluble in water, alcohol, and ether, but nearly insoluble in chloroform and benzene. Homogentisic acid is optically inactive and non-fermentable. Its aqueous solution has the properties of so-called alcaptonuric urine. It becomes greenish-brown from the surface downwards on the addition of very little sodium hydroxide or ammonia with access of oxygen, and on shaking it quickly becomes dark brown or black. It reduces alkaline copper solutions on slightly warming, and ammoniacal silver solutions immediately in the cold. It does not reduce alkaline bismuth solutions. It gives a lemon-coloured precipitate with Millon's reagent, which becomes light brick-red on warming. Ferric chloride gives to the solution a blue colour which soon disappears. On boiling with concentrated ferric chloride solution an odour of quinone develops. With benzoyl chloride and sodium hydroxide in the presence of ammonia we obtain the amide of dibenzoylhomogentisic acid, m. p. 204°, which can be used in the isolation of the acid from the urine, and also for its detection (Orton and Garrod, *J. Physiol.*, 1901, 27, 89). Among the salts of this acid must be mentioned the lead salt containing water of crystallisation and 34.79% Pb. This salt melts at 214 to 215°. For further discussion of properties see Mörner; *Upsala Lakara Föreningens Förhandlingar*, 17, 499.

In order to prepare the acid, heat the urine to boiling, add 5 gm. of lead acetate for every 100 c.c., filter as soon as the lead acetate has dissolved, and allow the filtrate to stand in a cool place for 24 hours until it crystallises (Garrod). The dried, powdered lead salt is suspended in ether and decomposed by H_2S . After the spontaneous evaporation of the ether the acid is obtained in nearly colourless crystals (Orton and Garrod). Briggs (*J. Biol. Chem.*, 1922, **51**, 453), has described a colorimetric method of determination.

Hydroxymandelic acid, $\text{C}_8\text{H}_8\text{O}_4$, *p*-hydroxyphenylglycollic acid, $\text{HO.C}_6\text{H}_4\text{CH(OH)COOH}$, is found in the urine in acute atrophy of the liver. The acid crystallises in silky needles. It melts at 162° , dissolves readily in hot water, less in cold water, and readily in alcohol and ether, but not in hot benzene. It is precipitated by basic lead acetate, but not by lead acetate.

Cynurenic acid (γ -hydroxy- α -quinoline-carboxylic acid), $\text{C}_{10}\text{H}_7\text{NO}_3$



has only been found thus far in dogs' and coyotes' urine, but not always; its quantity is increased by meat feeding. It does not occur in the urine of cats. Ellinger has been able to show positively that tryptophane is the mother substance of this acid.

The acid is crystalline, does not dissolve in cold water, but dissolves rather well in hot alcohol, and yields a barium salt which crystallises in triangular, colourless plates. On heating it melts and decomposes into CO_2 and cynurin. On evaporation to dryness on the water-bath with hydrochloric acid and potassium chlorate a reddish residue is obtained, which becomes first brownish-green and then emerald-green on adding ammonia (Jaffé's reaction). To isolate the acid from urine proceed as follows: Acidify with hydrochloric acid in the proportion 1:25. From the acid fluid both the uric acid and the cynurenic acid separate in the course of 24-48 hours. Filter off the crystalline deposit of the two acids, dissolve the cynurenic acid in dilute ammonia (uric acid is insoluble) and reprecipitate it with hydrochloric acid. (Capaldi *Z. physiol. Chem.*, 23), has proposed a method of estimating cynurenic acid quantitatively.

Oxyproteic Acids.—A series of nitrogenous sulphur-containing acids of doubtful nature and significance have been isolated by Bondzinski, Dombrowski and Panek (*Zeit. physiol. Chem.*, 1905, **46**, 83) from human urine, which are of interest because of their relationship to the proteins. They are antoxyproteic acid, containing 24.4% N and 0.61% S; oxyproteic acid, with 18.08% N and 1.12% S and alloxypoteic acid, with 13.55% N and 2.19% S.

Antoxyproteic acid is soluble in water, dextrorotatory and is precipitated from its concentrated solution by phosphotungstic acid. It does not give the protein colour reactions, but does give Ehrlich's diazo reaction. The salts of the alkalies, barium, calcium, and silver are soluble in water, whilst the barium and especially the silver salt is difficultly soluble in alcohol. The free acid and its salts are precipitated by mercuric nitrate or acetate even in strong acetic acid solution. Basic lead acetate does not precipitate the pure acid. According to Edlbacher (*Z. physiol. Chem.*, **127**) it is essentially of a peptide character, containing histidine which is responsible with phenols for the diazo reaction.

Oxyproteic acid does not give the Ehrlich diazo reaction or the xanthoproteic or the biuret reactions. It gives a faint Millon reaction and it is not precipitated by phosphotungstic acid. Oxyproteic acid is precipitated by mercuric nitrate or acetate in neutral reaction, but not by basic acetate. The salts are more readily soluble in water than the corresponding salts of the antoxyproteic acid. According to Freund and Sittenberger—Kraft (*Biochem. Z.*, **136**) it is sulphur free and yields urea and a volatile nitrogenous acid.

Alloxypoteic acid is soluble in water, does not give the biuret reaction or Ehrlich's diazo reaction and is not precipitated by phosphotungstic acid. It differs from the other two acids by being precipitated by basic lead acetate, and its salts are less soluble in alcohol than the other salts. According to Liebermann it is a mixture.

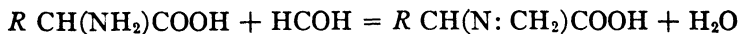
Uroferric acid is an acid isolated by Thiele (*Z. physiol. Chem.*, **37**, 251), which contains 3.46% S and has the formula $C_{35}H_{56}O_{19}N_8S$. This acid is readily soluble in water, saturated ammonium sulphate solution, and methyl alcohol. It is soluble with difficulty in absolute alcohol and insoluble in benzene, chloroform, ether and glacial acetic acid. It does not respond to either the biuret reaction or to Millon's, or Adamkiewicz's reactions. It is precipitated by mercuric nitrate and sulphate and also by phosphotungstic acid. Uroferric

acid has a rotation of $[\alpha]_D = -32.5^\circ$ at 18° . The existence of this acid is disputed by Bondzynski, Dombrowski and Panek and by Ginsberg.

For the quantitative estimation of the total oxyproteic acids in urine we refer to the complicated methods suggested by Ginsberg (Hofmeister's *Beiträge*, 1907, 10, 411) and by Gawinski (*Z. physiol. Chem.*, 1909, 58, 454).

Amino Acids.—Normal human urine contains from 0.4 to 1.0 grm. of total amino acid nitrogen per day, but only about one-fifth of this is free amino acid nitrogen. Under pathological conditions the amount may be considerable. The amino acid fraction seems to be raised in starvation and in disorders associated with tissue waste. By means of the naphthalenesulphochloride method the presence of glycocoll has been demonstrated in normal human urine, and this glycocoll does not exist free, but in a combination which is split by alkali. Various methods for the estimation of the total amino-acid nitrogen in urine have been suggested, but the method of Henriques (*Z. physiol. Chem.*, 1909, 60, 1) based upon Sørensen's (*Biochem. Z.*, 1908, 7, 45) formal titration and the method proposed by Van Slyke (*Proc. Soc. Exp. Biol. and Med.*, 1909, 7, 46) are to be recommended (see also pp. 304-316). Folin has developed a colorimetric method applicable to urine and blood (*J. Biol. Chem.*, 1922, 51, 393).

Henriques' Method.—This titration method is based upon the fact that if a neutral solution of formaldehyde is added to an amino acid solution, the amino groups form a methylene combination with the formaldehyde and make possible the determination, by simple titration, of the carboxyl groups present. The following equation explains the process:



50 c.c. of the urine are placed in a 100 c.c. graduated flask, and 1 c.c. of a 0.5% phenolphthalein solution and 2 grm. of solid barium chloride are added. After solution of the barium chloride, a saturated solution of barium hydroxide is added until the liquid is alkaline and then 5 c.c. more of the same solution (to remove the phosphates). The mixture is now made up to 100 c.c. with water, shaken well, allowed to stand for 15 minutes and filtered through a dry filter, 80 c.c. of the filtrate (corresponding to 40 c.c. of the urine) being

collected. This is placed in a 100 c.c. flask and the liquid neutralised with $N/5$ hydrochloric acid, litmus being used as indicator and then filled up to 100 c.c. with water. The ammonia is estimated in 40 c.c. (= 16 c.c. urine) by the Folin-Shaffer method or by any other method, and the other portion is used for the formaldehyde titration, according to Sørensen's suggestion.

The following solutions are necessary: 1. A solution of 0.5 gm. of phenolphthalein in 50 c.c. alcohol and 50 c.c. water. 2. A freshly prepared formaldehyde mixture, which consists of 50 c.c. of commercial formaldehyde (30 to 40%) to which 1 c.c. of phenolphthalein solution is added and then $N/5$ barium hydroxide solution until the solution has a faint pink colour. As a control solution 20 c.c. of boiled distilled water are placed in a flask, 10 c.c. of the above formaldehyde solution added, and then about 5 c.c. of $N/5$ barium hydroxide solution added from a burette, and the liquid re-titrated with $N/5$ HCl, added drop by drop, until the colour of the solution is just faintly pink (first stage). Then 1 drop of the barium hydroxide solution is added, when a pronounced pink colour should be the result (second stage). The urine is now titrated to this same colour by taking 20 c.c. of the solution and adding 10 c.c. of the formaldehyde mixture; then $N/5$ barium hydroxide solution is added to a deep red colour, then re-titrated with $N/5$ hydrochloric acid until the colour is fainter than the control solution, and finally barium hydroxide is added, drop by drop, until the colour is same as in the control solution. The number of c.c. of $N/5$ barium hydroxide actually used to neutralise the solution, when multiplied by 2.8, gives the mg. of nitrogen as amino-acid nitrogen, after subtracting the ammonia nitrogen, as especially determined. $N/5$ sodium hydroxide can be used instead of the $N/5$ barium hydroxide. Urea, creatine, creatinine and hippuric acid do not influence the results. If the formaldehyde titration differs before and after boiling with strong hydrochloric acid, it indicates the presence of polypeptides.

Method of Folin for Urine.—Dilute from 5–25 c.c. of urine to a volume of 25 c.c. in a 50 c.c. Erlenmeyer flask. Add 2 to 3 gm. of permutit and agitate very gently but continuously for 5 minutes. Decant the supernatant urine into another 50 c.c. flask. Add a further 2–3 gm. of permutit and shake as before for 5 minutes. By this double extraction with permutit every trace of ammonia is removed. Decant the supernatant urine into a flask or test tube.

It may be a little turbid, but this need not interfere with the determination. Put into test tubes graduated at 25 c.c. 1, 2, and 3 c.c., respectively, of a standard glycocoll solution in 0.1*N* hydrochloric acid, plus 0.2% of sodium benzoate. This standard solution should contain 0.1 mg. of glycocoll nitrogen per c.c. To these tubes add 1, 2, and 3 c.c., respectively, of 1% sodium carbonate solution.¹ (1 c.c. of sodium carbonate solution for each c.c. of 0.1*N* HCl present). Dilute the contents of each test tube to a volume of 10 c.c.

Transfer 5 c.c. of ammonia-free (usually diluted) urine to another test tube graduated at 25 c.c. Add 1 c.c. of 0.1*N* HCl and 1 c.c. of 1% sodium carbonate solution. Dilute to 10 c.c. Dissolve 250 mg. of the amino acid reagent (β -naphthaquinone monosodium sulphonate) in 50 c.c. of water, and add 5 c.c. of this solution to each standard and to the unknown urine.

Mix and set in a dark place over night. It is often desirable to take out the test tubes and inspect them after they have stood 10–15 minutes. If the test tube containing the urine appears much darker than the darkest standard, as may happen, especially in experiments planned to produce excessive amino acid excretion, then it is, of course, necessary to start another sample of urine, taking only 1, 2, or 3 c.c. and treating it in the same way as the first sample, not omitting to provide for a final volume of 15 c.c.

The following day the standard and the unknown sample or samples are first acidified by the addition of 1 c.c. of the special 25% acetic acid-acetate solution. To each are then added 5 c.c. of the 4% sodium thiosulphate solution. The contents of all tubes are diluted to a volume of 25 c.c. and, after mixing, the colour of the unknown is read against that of the standard having most nearly the same intensity of color.

¹ *Reagents Required for the Colorimetric Method.* (a) Standard amino acid solution. A solution containing 0.1% of nitrogen. For the blood determination the standard solution should contain 0.07% N. Glycocoll may be used or leucine, phenylalanine or tyrosine. Make up in 0.1*N* HCl containing 0.2% sodium benzoate.

(b) Special sodium carbonate solution. Dilute 50 c.c. of approximately saturated solution to 500 c.c. Titrate 20 c.c. of 0.1*N* HCl with the carbonate, using methyl red as an indicator. Dilute so that 8.5 c.c. are equivalent to 20 c.c. of 0.1*N* acid. The carbonate solution is about 1 per cent.

(c) Amino acid reagent. A 0.5% solution of the sodium salt of β -naphthaquinone-sulphonic acid freshly prepared. The quinone itself may be prepared according to directions given in the original paper.

(d) Special acetic acid-acetate solution. Dilute 100 c.c. of 50% acetic acid with an equal volume of 5% sodium acetate solution.

(e) Sodium thiosulphate solution. A 4% solution of the crystalline salt.

Method of Folin for Blood.—Into a large test tube graduated at 25 c.c. introduce 10 c.c. of blood filtrate (see Uric Acid Method p. 409). To another test tube transfer 1 c.c. of the standard amino acid solution and 8 c.c. of water. Add 1 drop of phenolphthalein solution to each. Add 1 c.c. of special 1% sodium carbonate solution to the standard, and to the blood filtrate add the carbonate solution, drop by drop, until the same pink colour as in the standard is obtained (6–8 drops are usually required). Add 2 c.c. of freshly prepared 0.5% β -naphthaquinone-sulphonic acid solution to each, mix, and set aside over-night in a perfectly dark place. The next day add first 2 c.c. of acetic acid-acetate solution and then 2 c.c. of 4% thiosulphate solution to each tube. This decolorises the excess of reagent. Dilute to the 25 c.c. mark, mix, and make the colour comparison. The reading of the standard over the reading of the unknown, times 7 gives, mg. amino-acid nitrogen in 100 c.c. of blood. Normally 5–8 mg. are found.

Acids of Bile

The bile contains certain conjugated acids which are strictly peculiar to that secretion. They occur as sodium salts, and are not found in the pancreatic juice, or in other normal animal secretions. These bile salts, by reducing the surface tension of the intestinal contents, assist in the emulsification of fats and thus improve fat digestion. They also assist in the absorption of fatty acids, forming clear, diffusible solutions of the latter up to an acidity of pH 6.2, whereas alkaline soaps appear to be absorbed only in a reaction of pH 9, or greater, which does not commonly occur in the intestines (Verzár and Kúthy. *Biochem. Z.*, 1929, 205, 369).

The secretion of bile salts is increased by feeding with protein, particularly with meat or liver protein. Tryptophane appears to be one of the important amino acids concerned in this effect (Smith and Whipple: *J. Biol. Chem.*, 1928, 80, 659–697).

Human bile is a dark golden yellow or yellow-brown liquid having an odour like that of musk, a very bitter taste, and a faintly alkaline reaction. In other animals, such as the ox, it may have a more greenish colour. The sp. gr. averages about 1.020. In its original condition, bile rapidly putrefies, but if the secretion is diluted, acidified with acetic acid, and filtered from the precipitated mucin, etc., it may be readily preserved.

Bile is a secretion of a very variable character. The following analyses show the general composition of the human liver bile in 1,000 parts:

Solids.....	25.200	35.260	170.32
Water.....	974.800	964.740	829.68
Mucin and pigments.....	5.290	4.290	41.91
Bile salts.....	9.310	18.240	96.97
Taurocholate.....	3.034	2.079	27.40
Glycochocolate.....	6.276	16.161	69.57
Fatty acids besides soaps.....	1.230	1.360	9.86
Cholesterol.....	0.630	1.600	2.23
Lecithin.....		0.574	1.9
Fat.....	0.220	0.956	11.17
Soluble salts.....	8.070	6.760	2.88
Insoluble salts.....	0.250	0.490	2.22

These figures are given by Hammarsten. Those in the third column are for gall bladder bile.

A complex and concentrated solution such as bile is very apt to form deposits under abnormal conditions. Hence arise the well-known concretions called biliary calculi and gall-stones.¹

The two chief acids of bile are glycocholic and taurocholic acid. The former of these is the more abundant in the human and ox-bile, in the proportion of fully 3 to 1; but is replaced by taurocholic acid in the bile of the dog and carnivora generally. Other bile-acids of less frequent occurrence and abundance are also met with (page 454). This follows from the fact that there are several cholic acids. Hammarsten found a third type of bile acid in the bile of the shark, yielding sulphuric acid and a cholic acid on hydrolysis, which he called scymnol.

¹ **Biliary Calculi.**—Under this denomination are comprised all those concretions which are formed in the bile. They are found in all parts of the biliary apparatus, occurring most frequently in the gall-bladder or gall-ducts, but sometimes in the intestinal canal. Their size varies from very small granules to (occasionally) that of a pigeon's egg. The form is generally oval, but when several calculi occur together in the gall-bladder, facets are generally formed by their mutual attrition. The colour of biliary calculi ranges from nearly white to yellow, brown, and dark green. Gall-stones are generally brittle, and can be readily reduced to a powder having a greasy feel.

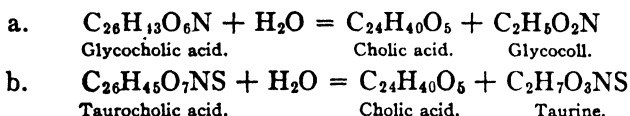
Gall-stones usually contain cholesterol as their leading constituent, calcium carbonate and bile-pigments being also present in very variable proportion. Fats, silica, uric acid, and compounds of iron, zinc, copper, and manganese have been observed as occasional constituents. Sometimes the bile-pigments preponderate, occasionally amounting to 60% of the calculus. Besides bilirubin and biliverdin, there have been found in gall-stones: biliprasin, $C_{42}H_{52}O_8N_4$, bilifuscin, $C_{42}H_{46}O_8N_4$, bilicyanin, bilihumin, etc. The bilirubin exists as a calcium salt, $Ca(C_{42}H_{40}O_8N_4)_2(?)$, which circumstance prevents the solution of the colouring matter in chloroform unless the stone be previously treated with acid. On boiling powdered gall-stones with alcohol or ether, cholesterol is almost the only constituent dissolved. Dilute hydrochloric acid will subsequently dissolve the calcium, whether existing as carbonate or as the bilirubin compound, and chloroform will then dissolve the bilirubin and bilifuscin. Subsequent boiling with alcohol will dissolve biliverdin and biliprasin, while bilihumin remains insoluble.

Biliary calculi are usually saturated with bile, which has become desiccated after removal from the organism. The nucleus generally consists of mucus.

For the preparation of the mixed sodium salts of the bile-acids, ox-bile should be mixed with washed sand, and evaporated at 100° till the residue can be powdered. The product is then extracted with boiling absolute alcohol, which dissolves the salts of the bile acids, while leaving pigment, mucin, and a portion of the inorganic salts undissolved. The green alcoholic solution is filtered and boiled with animal charcoal till colourless,¹ when it is again filtered, the residue taken up in a minimum quantity of absolute alcohol, and ether added until a permanent turbidity is produced. On standing for a few hours the mixed sodium salts of glycocholic and taurocholic acids will be deposited as a white, semi-crystalline mass known as "Plattner's crystals," which should be pressed between blotting paper and dried.

From the sodium salts, prepared as above, free glycocholic acid may be readily obtained by dissolving the crystals in a little water, adding ether and then dilute sulphuric acid as long as a precipitate is produced. On stirring, glycocholic acid separates as a crystalline mass of shining needles, while the very soluble taurocholic acid remains in solution.

Both glycocholic and taurocholic acid generally undergo hydrolysis under the influence of dilute acids or alkalis. In each case one of the products of the reaction is cholic acid. In the case of glycocholic acid, the second product is glycocoll. The following equations express the reactions:



These changes occur naturally in the intestine. In a state of health by far the larger proportion of the products is re-absorbed, and passes back into the liver.

Glycocholic acid, $\text{C}_{26}\text{H}_{43}\text{O}_6\text{N}$, or $\text{C}_{23}\text{H}_{39}\text{O}_3\cdot\text{CO}\cdot\text{NH}\cdot\text{CH}_2\text{COOH}$. This acid was first described by Gmelin, in 1826, under the name of cholic acid. It occurs as a sodium salt in human and ox-bile to the extent of 3 to 5%, together with more or less of the analogous taurocholic acid. The bile of the herbivora generally contains glycocholic acid, but that of the carnivora contains taurocholic acid with mere traces of glycocholic acid.

¹ An alternative plan is to mix the original bile into a paste with animal charcoal, dry the mixture at 100° , and exhaust it with boiling absolute alcohol.

Glycocholic acid may be readily prepared by the process described on page 452. By pressing the crystals, and recrystallising the acid from hot water, it is obtained perfectly pure. It has also been prepared synthetically by Bondi and Müller (*Z. physiol. Chem.*, 1906, 47, 499) by the action of nitrous acid upon cholalic acid hydrazide and then the conjugation of glycocoll with the azide thus obtained.

Marshall prepared glycocholic acid by treating fresh bile with a little hydrochloric acid, and filtering from the precipitate of mucin, etc.; 100 volumes of the filtrate are then treated with 5 volumes of ether, and the mixture shaken and allowed to stand for some hours.¹ The crystals of glycocholic acid which form are filtered off, washed with water containing ether and hydrochloric acid, dried in the air, and recrystallised from hot water.

Glycocholic acid forms fine glistening needles, or prisms, which taste at first sweet and afterward bitter. The m.p. varies depending upon the method of preparation. Emich found the melting-point for the acid crystallised from water to be 132 to 134°. Hammarsten found the acid crystallised from alcohol by water addition and dried at 100° to melt at 126–128°. It is about as strong an acid as lactic acid.

Glycocholic acid is soluble in about 300 parts of cold or 120 parts of boiling water, and is very soluble in alcohol; but is very slightly soluble in ether, and practically insoluble in chloroform and benzene. Glycocholic acid forms salts which are extremely soluble both in water and in alcohol, but very slightly soluble or insoluble in ether. An alcoholic solution of the free acid has a rotation of $[\alpha]_D = +29.0^\circ$ and the rotation of the sodium salt is $+25.7^\circ$. On boiling with water it is converted into paraglycocholic acid, an isomeric form.

Sodium glycocholate, $\text{NaC}_{26}\text{H}_{42}\text{O}_6\text{N}$, forms stellate needles. Potassium glycocholate occurs in the bile of certain fishes.

When dissolved in warm concentrated sulphuric acid (or, according to Strecker, by simply heating above 100°), glycocholic acid loses the elements of water, and is converted into glycocholonic acid, $\text{C}_{26}\text{H}_{41}\text{O}_5\text{N}$, a body forming an insoluble barium salt, but possessing nearly the same optical activity as the parent acid.

¹ F. Emich (*Monats. Chem.*, 1882, 3, 325) recommends the use of benzene instead of ether in this process, and states that bile which gives no precipitate with ether readily gives crystals when benzene is employed.

As stated on page 450, when boiled with dilute acids or alkalis, glycocholic acid undergoes hydrolysis with formation of cholic acid and glycocoll. The reaction is analogous to the formation of benzoic acid and glycocoll from hippuric acid.

Glycholeic acid, $C_{26}H_{43}O_6N$, is a second glycocholic acid isolated from ox-bile by Wahlgren (*Z. physiol. Chem.*, 1902, **36**, 556), and which also occurs in human bile. Wieland and Stender (*Z. physiol. Chem.*, **106**), have synthesised a closely related compound. It crystallises in short prisms or needles and is insoluble in cold water and slightly soluble in boiling water. It is soluble in alcohol, but not very soluble in ether or acetone, while it is insoluble in benzene and chloroform. Glycholeic acid, m. p. 175 to 176° , gives a brownish-yellow solution with a strong green fluorescence with concentrated sulphuric acid. On hydrolytic cleavage it yields glycocoll and choleic acid, which is apparently a combination of desoxycholic acid and fatty acid. The alkali salts are soluble in water and are more readily precipitated by neutral salts (NaCl) than the glycocholates.

The principle of the preparation of the pure glycocholic acids consists in treating a 2 to 3% solution of bile free from mucus, when rich in glycocholic acid, with ether and then with 2% hydrochloric acid. If the bile is not directly precipitable with hydrochloric acid (bile relatively poor in glycocholic acid), then the chief mass of the glycocholic acid is first precipitated with ferric chloride, or better, with lead acetate, the precipitate decomposed with sodium hydroxide, and the 2% solution treated, as above stated, with ether and hydrochloric acid. The crystalline and washed mass is boiled with water and, on cooling, glycocholic acid crystallises out and then this is recrystallised from water or from alcohol by the addition of water. The residues that remain after boiling in water (paraglycocholic acid and glycholeic acid) are converted into barium salts, and after a complicated process the glycholeic acid is obtained.

Taurocholic Acid.— $C_{26}H_{45}O_7NS$, or $C_{23}H_{39}O_3.CO NH CH_2CH_2SO_2OH$. This acid is a constituent of the bile of all carnivora, and exists in human bile together with glycocholic acid. The preparation of pure taurocholic acid from human or ox-bile is difficult, since the portion of the glycocholic acid which remains in solution with the more readily soluble taurocholic acid is very troublesome to separate therefrom. Hence it is preferable to prepare taurocholic acid from dog's bile, which should be treated by the process described on page

450 for the preparation of "Plattner's crystals." The sodium salt is dissolved in water and treated with ferric chloride. The solution is filtered and the filtrate nearly neutralised with NaOH. The treatment with ferric chloride and NaOH is repeated and the precipitates are combined for the preparation of taurocholic acid. The filtrate is made faintly alkaline, filtered, saturated with sodium chloride and filtered. The precipitate is dissolved in water and again saturated with NaCl. After separation from NaCl by alcohol treatment, the precipitate is dissolved in the smallest possible amount of alcohol, and alcoholic sulphuric acid solution is added to 2-3% acid. The liquid is filtered and the free acid precipitated with ether. The precipitate is dissolved in alcohol, and water is added until turbidity appears, and the acid allowed to crystallise.

Taurocholic acid may be isolated from the ferric precipitate (Abderhalden: *Handb. Biol. Arbeitsmeth.*, Abt. I, Teil 6, p. 221).

The amount of taurocholic acid present in bile may be estimated without isolating it, by calculation from the amount of sulphur contained in the alcoholic extract of the bile, since no other sulphuretted substance passes into the alcoholic solution. For this purpose the dried alcoholic extract from a known quantity of bile is evaporated to dryness on a water-bath with fuming nitric acid, by which treatment the sulphur is converted into sulphuric acid. The residue is taken up with water, and the solution precipitated with barium chloride. One part of BaSO₄ corresponds to 2.16 parts of taurocholic acid.

Taurocholic acid forms deliquescent silky needles, very soluble in water and in alcohol, but insoluble in ether, benzene, and acetone. The same ready solubility characterises the salts of taurocholic acid except the precipitate produced by basic lead acetate in presence of ammonia, which is insoluble in water, but soluble in boiling alcohol. Taurocholic acid has the ability to hold the insoluble glycocholic acid in solution.

Taurocholic acid and its salts are dextrorotatory, the value of $[\alpha]_D$ for the solution of the sodium salt in alcohol being $+24.5^\circ$. On heating, taurocholic acid contracts at 140° , begins to decompose at 160° , and melts at 180° to a brown liquid.

By boiling with dilute acids or alkalis, taurocholic acid is hydrolysed with formation of cholic acid and taurine (page 450). The same decomposition occurs on merely boiling an aqueous solution of taurocholic acid, and takes place naturally in the intestine. This

behaviour accounts for the absence of unchanged taurocholic acid from the urine, in which it is represented by taurine and taurocaramic acid.

Taurocholic acid possesses the power of completely precipitating albumins and globulins from their solutions, but it does not precipitate peptones. It is stated to possess powerful antiseptic properties.

Taurocholeic acid, $C_{26}H_{45}O_6NS$ is found in dog-bile and ox-bile. It is amorphous, readily soluble in water and in alcohol, but insoluble in ether, acetone, chloroform, and benzene. The soluble alkali salts can be salted out by sodium chloride. On cleavage taurocholeic acid yields taurine and choleic acid.

Hyoglycocholic acid, $C_{26}H_{43}O_5N$, the crystalline glycocholic acid from pig's bile, is characterised by insolubility in water, intense bitter taste and by being precipitated by calcium, barium and magnesium chlorides. According to Windaus and Bohne it is a combination of glycocoll with a desoxycholic acid, hence glycohyodesoxycholic acid.

Cheno-taurocholic acid, is the chief taurocholic acid of goose-bile. It is a taurodesoxycholic acid.

The Cholalic Acids.—The best studied of the cholalic acids are cholic, desoxycholic, and lithocholic acids.

Cholic acid $C_{24}H_{40}O_5$ or $C_{23}H_{36}(OH)_3COOH$ is the acid product of the hydrolysis both of glycocholic acid and taurocholic acid, the former yielding glycine and the latter taurine as the basic product of the decomposition (page 450).

Cholic acid occurs in the small and large intestines as a product of the decomposition of the bile-acids. It is also present in the fæces of men and lower animals.

For the preparation of cholic acid, ox-bile should be boiled for 24 hours, in a flask fitted with a reflux condenser, with as much caustic baryta as it will take into solution. The liquid is filtered while still hot, and the filtrate concentrated until it yields a copious crop of crystals of barium cholate. The salt is recrystallised from boiling water, decomposed by hydrochloric acid, and the free cholic acid crystallised from a small volume of boiling alcohol. Or the acid may be dissolved in sodium hydroxide solution containing a little ether, and the solution acidulated with hydrochloric acid. The crystals which form after a time are separated and treated with ether, which is

poured off after half an hour, and the residue dissolved in boiling alcohol. Water is gradually added to this solution till a permanent precipitate appears, when cholic acid will crystallise out on cooling.¹

Cholic acid crystallises as rhombic plates or prisms with 1 molecule of water of crystallisation and in large rhombic tetrahedra or octahedra with 1 molecule alcohol of crystallisation. The crystals are very sparingly soluble in water, requiring 750 parts even of the boiling solvent, but are soluble in alcohol and soluble with difficulty in ether. The alcohol of crystallisation is driven off by heating to 100 to 120° for a long time and the acid free from water and alcohol melts at about 195°. The specific rotation of the crystalline acid is $[\alpha]_D = +31.55^\circ$ and $[\alpha]_D + 37.02^\circ$ for the anhydrous acid. Cholic acid gives a blue compound with iodine, and if the finely powdered acid is added to 25% hydrochloric acid at the ordinary temperature, a beautiful violet-blue coloration gradually appears, and on allowing to stand it gradually changes to green and yellow. The blue solution shows an absorption band at D.

The alkali salts are readily soluble in water and less soluble in alcohol. Barium cholate forms fine silky needles (often radiated) very soluble in boiling water and in alcohol. The sodium and barium salts of the cholic acid isolated from human bile are less soluble than the corresponding compounds prepared from ox-bile. The difference has been attributed to the non-identity of the cholic acid from the two sources, but it appears to be really due to contamination of the cholic acid from human bile with a small quantity of the analogous fellic acid. The cholic acids from the bile of several other animals also exhibit certain differences, which in some cases at least are due to the presence of associates of ordinary cholic acid. Thus:

Choleic acid, $C_{24}H_{40}O_4$, is obtained in small quantity, together with cholic acid, when the latter is prepared from ox-bile. It is believed to be an addition product of desoxycholic acid with higher fatty acid

¹ The same method may be employed for the estimation of the cholic acid obtainable from bile, but for this purpose Lassar-Cohn (*Ber.*, 1893, 26, 146) recommends the following process: 20 c.c. of the bile is mixed with 2 gm. of sodium hydroxide, the liquid boiled for 24 hours, saturated with carbon dioxide, and evaporated to dryness at 100°. The residue is boiled with nearly absolute alcohol, until free from the salts of organic acids which are only sparingly soluble in water, and the solution, after dilution with 4 measures of water, is precipitated 0.5 gm. of barium chloride in dilute solution. The filtered liquid is acidified with hydrochloric acid, and shaken with ether. This, in presence of alcohol, readily extracts the cholic acid, which is obtained on evaporating the etherealalcoholic solution. Lassar-Cohn obtained the following percentage of acids from a sample of ox-bile treated in the above manner: Cholic acid, 4.790; choleic acid, 0.085; myristic acid, 0.004; stearic and palmitic acids, 0.146; resinous acids, 0.120; and loss, 0.050 %.

in the molecular proportions of 8:1. The compound is so stable that it may be recrystallised with constant melting point (186°), and enters as such into combination with glycocoll. It is typical of a class of compounds undoubtedly of great biological importance. As different fatty acids may be present in such compounds the properties must vary somewhat. Those given are for preparations obtained from bile. It is soluble at 20° in 22,000 parts of water, in 750 of ether, in 14 parts of absolute alcohol, and in 25 parts of alcohol of 75%. Barium choleate dissolves in 1,200 parts of cold water, the solubility rapidly increasing with the temperature. The specific rotation of the alcoholic solution with a concentration of 2.49% is $[\alpha]_D = +48.87^{\circ}$. The barium salt which crystallises in spherical aggregations of needles from hot alcohol is less soluble in water than the corresponding cholic acid salt.

Desoxycholic acid, $C_{24}H_{40}O_4$ or $C_{25}H_{37}(OH)_2COOH$ was first obtained, together with cholic acid, from bile which had been allowed to putrefy for some time. Desoxycholic acid differs from cholic acid in its intense bitter taste, its ready solubility in alcohol, and in its sparing solubility in glacial acetic acid, and it does not give any blue iodine compound, nor does it give the colour reaction with hydrochloric acid. Its barium salt is soluble with difficulty in cold water, but dissolves in boiling alcohol. The acid, crystallised from a mixture of alcohol and ether, melts at 153 to 155° . Completely freed from solvent it has a m. p. of 172° . Its specific rotation in alcohol ($C = 2.034\%$) is $(\alpha)_D^{20} = +57.02$.

Hyodesoxycholic acid, $C_{24}H_{40}O_4$, and *chenodesoxycholic acid*, $C_{24}H_{40}O_4$, are obtained by the hydrolysis of the conjugated acids of the bile of the pig and the goose, respectively. The latter is also found in human bile and is also called anthropodesoxycholic acid. Both are isomers of desoxycholic acid.

Lithocholic acid, $C_{24}H_{40}O_3$ or $C_{25}H_{38}(OH)COOH$ occurs in small amounts in bile and gall stones of the ox. It is readily soluble in hot alcohol and more soluble in ether than the other cholalic acids. It forms crystals m. p. 185 – 186° , $[\alpha]^{19} = +23$ at 33° in absolute alcohol, $C = 1.54390$.

The following is a tabular scheme for the separation of bile-acids:

SCHEME FOR THE SEPARATION OF BILE ACIDS.

Dissolve the sodium salts, precipitated by ether, in water and precipitate the solution by neutral lead acetate.

<p>Precipitate contains the lead salts of choleic and glycocholic acids. Boil with alcohol, and evaporate the solution to dryness with sodium carbonate. Take up with alcohol, and precipitate the filtered liquid with ether; both acids give crystalline sodium salts. Sodium glycocholate forms six-sided prisms, with a single face having very oblique truncations. Agitate the liquid with dilute sulphuric acid and ether, and filter.</p>	<p>Filtrate contains taurocholic acid. Add a solution of basic lead acetate and ammonia, wash and convert the precipitate to the sodium salt. Boil the latter for six hours with hot saturated baryta-water. The taurocholic acid is hydrolysed, with the formation of <i>taurine</i> and <i>cholic acid</i>. Filter the boiling liquid, and pass carbon dioxide through the filtrate. Filter again, and add hydrochloric acid to the filtrate.</p>		
<p>Precipitate may contain <i>cholic</i> and <i>glycocholic</i> acids. Choleic acid may be recognised by its characteristic resinous appearance, and by its precipitation as a resin on acidifying a solution of one of its salts.</p>	<p>Filtrate may contain <i>cholic acid</i>, which may be recognised by its crystalline form, and by the characters of its barium salt.</p>	<p>Precipitate consists of <i>cholic acid</i>.</p>	<p>Filtrate. Add sufficient sulphuric acid to precipitate any remaining barium. The excess of sulphuric acid is then removed by addition of lead hydroxide, and any lead which may have dissolved is precipitated by passing hydrogen sulphide. The solution is finally evaporated to dryness on the water-bath, and the residue taken up with alcohol. Any undissolved matter is <i>taurine</i>.</p>

When it is merely desired to ascertain the amounts of glycocholic and taurocholic acids in a mixture of their sodium salts, this can be effected by oxidising a weighed quantity of the mixture with fuming nitric acid, and converting the resulting sulphuric acid into barium sulphate, as described on page 453. 100 parts of BaSO_4 represents 225.3 parts of sodium taurocholate, and by deducting the amount thus found from that of the mixed sodium salts the weight of sodium glycocholate may be obtained.

Preparation of Cholic, Desoxycholic and Choleic Acids.—Remove mucin from bile with alcohol and prepare a 5% aqueous bile salt solution. Add one fourth the volume of 30% NaOH. Boil for 30 hours in an iron vessel, most simply in a large autoclave. Neutralise most of the alkali, and, after filtration, precipitate the crude salts with HCl. Dissolve in alcohol. Add syrupy NaOH solution to faint alkaline reaction. Heat on the water-bath 1–2 hours beneath a reflux condenser. Sodium cholate crystallises out. Filter hot and wash with boiling alcohol. Dissolve the salt in water, add excess of acid and two volumes of alcohol. Cholic acid crystallises out.

Drive off the alcohol from the alkaline filtrate obtained as above. Dissolve in water and precipitate with HCl. Dissolve in ammonia and precipitate with 20% barium chloride. Dissolve the precipitate in cold alcohol diluted with a little water. Filter from the residue, warm and add excess NaOH, evaporate, pulverise and extract with alcohol. Drive off alcohol and dissolve in water. Precipitate free acids with HCl. Wash, press and dry in air.

Rub up the precipitate thoroughly with acetic acid. Filter on a Buchner funnel and repeat the process. Recrystallise the residue from acetic acid. An acetic acid compound of desoxycholic acid (m. p. 144–145°) is obtained. Drying for several days in a high vacuum at 130° is required to remove the acetic acid. If recrystallised from alcohol, a temperature of 110° is sufficient.

Choleic acid is prepared from desoxycholic acid by dissolving the latter in hot alcohol with the proper amount of stearic acid and allowing it to crystallise. It is recrystallised from 4½–5 times its bulk of alcohol. It is identical with natural choleic acid.

Lithocholic acid comes down with the barium desoxycholate, from which it can be separated by a laborious process (Wieland and Weyland: *Z. physiol. Chem.*, 110).

Anthropodesoxycholic acid. For the preparation of the acid see Wieland and Revery: (*Z. physiol. Chem.*, 140).

Hyodesoxycholic acid. For its preparation see Windaus and Bohne: (*Annalen*, 433).

Pettenkofer's Reaction for Bile-acids.—The most delicate and characteristic reaction of cholic acid is that known as Pettenkofer's test, but which could be more appropriately termed the furfural reaction. It depends on the vivid purple coloration produced on treating cholic acid with strong sulphuric acid and furfural or any substance (such as sugar) capable of yielding furfural by reaction with the acid. The reaction is common to all of the cholic acids, and also to the conjugated forms in which they exist in bile from various sources. As the detection of bile, especially in urine, is often of considerable pathological importance, the reaction has a practical interest.

Pettenkofer's reaction is most simply observed by treating a drop of bile on a porcelain surface with a drop of a 10% solution of cane-sugar and adding a drop of strong sulphuric acid. A bright cherry-red colour will be produced, and, either at once or on gently warming

the mixture, will rapidly change to a magnificent purple tint, ultimately becoming bluish. Too high a temperature must be carefully avoided, or the reaction will be obscured by the charring of the sugar, and excess of sugar should be avoided for the same reason. Hence it has been proposed to employ furfural instead of sugar in important cases. The red solution shows an absorption spectrum of two bands, one at F and the other between D and E near E. The sulphuric acid used should be, as far as possible, free from SO_2 and oxides of nitrogen.

In using furfural, 1 c.c. of the liquid to be tested, which may be either aqueous or alcoholic, is treated in a test-tube with 1 drop of a solution of furfural in 1,000 parts of water, 1 c.c. of concentrated sulphuric acid is then added, and the tube immersed in water so that the temperature does not exceed 50 to 60°.

The tendency to char which attends the use of cane-sugar may be avoided by employing dextrose in its place. If a little dextrose is dissolved in concentrated sulphuric acid, and a few drops of the freshly made reagent are allowed to fall in the centre of a small pool of urine on a white plate, the play of colours produced in the presence of bile acids may be observed under very favourable conditions.

It has also been proposed to employ phosphoric acid in place of sulphuric acid, but the substitution is not desirable.

Unfortunately, Pettenkofer's reaction is not peculiar to the bile acids. Udranszky has enumerated 76 organic substances which behave somewhat similarly, but of these, only α -naphthol gives the reaction as readily as the bile acids. A useful confirmation of the reaction is afforded by the absorption spectrum of the colouring matter. For this purpose the colour should be produced as already described, and the purple liquid diluted with glacial acetic acid or alcohol until the tint is of suitable depth for observation of the spectrum. The colouring matter from bile acids exhibits four absorption bands. Of these, the band slightly on the red side of the Fraunhofer line E and another about F, are the best defined. Two others may be observed near D. On further dilution of the liquid, these two bands disappear entirely, and that between D and E becomes indistinct, but the most refrangible band still persists. The cherry-red colour produced by albumin, when treated with sulphuric acid and sugar, shows one absorption band, between E and F, and

does not exhibit the dichroism characteristic of the colouring matter of the bile acids.

The colouring matter formed in Pettenkofer's reaction is soluble in ether.

Pettenkofer's reaction is obscured or actually falsified by proteins, fatty matters and certain colouring and extractive matters, and hence it is important to remove these before employing the test. In applying the test to urine, purification is in some cases sufficiently effected by rendering the liquid distinctly but not strongly acid with acetic acid, boiling for a minute or two, and filtering from any mucus, albumin, etc., which may be precipitated.

In applying Pettenkofer's test to urine, it is often desirable to concentrate the liquid previously on the water-bath. A little cane sugar or glucose is then dissolved in it, and a portion of the cold liquid placed in a test-tube. Strong sulphuric acid is then allowed to run down the side of the tube so as to form a distinct layer below the urinous liquid, when the characteristic purple coloration will be developed at the junction of the two strata if any bile acids are present. An alternative and very delicate mode of performing the test is to dip a slip of filter paper in the sweetened urine, and allow it to dry spontaneously. When dry, a drop of concentrated sulphuric acid is applied to the paper by means of a glass rod, when if bile acids are present even to the extent of 0.03%, in less than half a minute a violet stain will be produced on the paper, which is best viewed by transmitted light.

In many cases it is necessary previously to isolate the bile acids from the urine in an approximately pure condition before applying Pettenkofer's test. For this purpose 500 c.c. or more of urine should be used. Evaporate nearly to dryness. If albumin is present first render slightly alkaline so that the protein will not coagulate. Extract with 95% alcohol. Evaporate to dryness and take up with, absolute alcohol. Filter, evaporate, take up with water. Add weak ammoniacal lead acetate, avoiding excess. Leave over night. The precipitate is filtered off, washed well, pressed and boiled with alcohol, which dissolves the lead salts of the bile acids, leaving the urate, phosphate, etc., insoluble. The alcoholic liquid is filtered boiling hot, and evaporated on the water-bath with a few drops of a solution of sodium carbonate. From the residue the sodium salts of the bile acids are dissolved by absolute alcohol, and to the solution Pettenkofer's and other tests can be advantageously

applied. Frequently it is necessary to precipitate again with lead acetate and to repeat the above process. To detect traces of bile acids, the alcoholic solution of the sodium salts is concentrated to a few drops, and three or four drops of dilute sulphuric acid (1:4) added, together with a minute quantity of cane sugar or dextrose. The liquid is then evaporated at a gentle heat, when the characteristic violet coloration will be produced with as little as 0.0001 grm. of bile acids.

To obtain the bile acids from blood or serous fluids sufficiently pure so that Pettenkofer's test can be applied to them, the protein and fat must first be removed. The protein is removed by making the liquid first neutral and then adding a great excess of alcohol, so that the mixture contains at least 85% of water-free alcohol. After filtration the precipitated protein is extracted with fresh alcohol, all filtrates united, the alcohol distilled, and the liquid evaporated to dryness. The residue is completely exhausted with strong alcohol, filtered, and the alcohol entirely evaporated from the filtrate. The residue is extracted with ether and dissolved in water, and filtered if necessary, and the solution precipitated by basic lead acetate and ammonia. The washed precipitate is dissolved in boiling alcohol, filtered while warm, and a few drops of sodium hydroxide solution added. After evaporation to dryness, the residue is extracted with absolute alcohol, filtered, and an excess of ether added. The precipitate now formed may be used for Pettenkofer's test. It is not necessary to wait for crystallisation; but one must not consider the crystals which form in the liquid as being positively crystallised bile. It is also possible for needles of alkali of ether added. The precipitate now formed may be used for acetate to be formed. In this connection it must be remarked that a confusion with phosphatides, which also give Pettenkofer's reaction, is not excluded, and a further testing and separation is advisable. It must be remembered that while Pettenkofer's reaction is given by all the acids of bile, and by the acid products of their hydrolysis, no similar coloration is produced by taurine or by biliary pigments (see below). Medical men often fall into error on this point, and assume the absence of bile pigments from urine because the sample gives a negative reaction with Pettenkofer's test. It is a fact that in jaundice the urine contains very little bile acids, and frequently they are entirely absent, while, on the other hand, the bile pigments are conspicuously present in the urine of jaundiced persons.

Bang suggests a simpler procedure. Add to 20–50 c.c. of urine 2–3 drops of serum. Saturate the warm solution with magnesium sulphate. Acidify with 2 drops of 25% HCl. Press the filter with precipitate between papers and then boil in a test-tube with 10–15 c.c. of alcohol. Transfer the solution to another tube, add powdered baryta, boil and filter. The filtrate should have not more than a slight yellow colour. Evaporate. To the residue add a drop of 1% sucrose and 2–3 c.c. of fuming HCl. Bile salts are indicated by a red-violet colour. Pettenkofer's test may also be applied.

For the detection of bile acids in fæces see methods given in Abderhalden: *Handb. Biol. Arbeitsmethoden*, Abt. 1, Teil 6, p. 247, 1925.

The test of Hay for bile acids is based on the reduction of surface tension of solutions produced by bile salts. Cool urine to 17° or lower and sprinkle finely pulverised sulphur over the surface. If bile salts are present the sulphur sinks. In the absence of other substances reducing surface tension the test is very delicate.

A quantitative Pettenkofer test applicable to the determination of bile acids in blood is suggested by Aldrich and Bledsoe (*J. Biol. Chem.*, 1928, 77, 519).

For method of determining bile acids in bile see Foster and Hooper (*J. Biol. Chem.*, 1919, 38, 355).

Gregory and Pascoe (*J. Biol. Chem.*, 1929, 83, 35) find earlier methods unsatisfactory and suggest the use of a special colour reaction and monochromatic light. They find no bile acid in normal blood. To a dilute bile acid solution there is added 34 volume per cent of H₂SO₄ and 0.05 volume per cent of furfural and the mixture heated for 30 minutes at 65°. A blue colour results. The reaction is more specific than Pettenkofer's test.

Bile Pigments

Bile contains certain colouring matters, which are derived from the hæmatin radicle of hæmoglobin¹ and are not chemically related to the bile acids.

Bilirubin, C₃₂H₃₆O₆N₄, is the yellow pigment of the bile of man and herbivorous animals. It is troublesome to prepare pure,

¹ The bile pigments contain no iron, which, however, exists in the bile in the form of phosphate, and is deposited in comparatively large amount in the liver in cases of pernicious anæmia.

but is best obtained by extracting powdered human or ox gall stones with ether, which extracts cholesterol. The residue is boiled with water, and treated with dilute hydrochloric acid to decompose the calcium salt, which is the form in which bilirubin occurs in gall-stones. The mass is washed, dried, and extracted with chloroform, the chloroform distilled off, and the residue treated with absolute alcohol. It is then again dissolved in chloroform, and precipitated by absolute alcohol. It may be further purified by crystallising from methyl alcohol saturated with ammonia as the ammonium salt. Recrystallisation from pyridine gives the free bilirubin. For further details see Abderhalden (*Handb. Biol. Arbeitsm.*, Abt. 1, Teil 8).

Bilirubin is an orange powder, insoluble in water, alcohol, or ether, but soluble with some difficulty in benzene and chloroform. It acts as a weak dibasic acid. It dissolves in alkalis, with orange colour, and is precipitated unchanged if hydrochloric acid is at once added, and may be extracted by agitation with chloroform; but if the alkaline liquid is exposed to the air it gradually absorbs oxygen, and then yields a green precipitate of biliverdin, $C_{33}H_{36}N_4O_8$, when acidified. It can be obtained as crystals from its solution in hot dimethylaniline. If the oxidation is carried further, as by adding yellow nitric acid to an alkaline solution of bilirubin mixed with an equal measure of alcohol, a blue pigment, bilicyanin, is formed; next a violet, which is perhaps a mixture of the red and blue; then a red colouring matter; and lastly a yellow pigment, called by Maly *choletelin*.

The foregoing colour reactions may be conveniently observed by spreading a drop of bile in a thin film on a porcelain plate, and placing a drop of yellow nitric acid in the centre, when a series of rings will be produced, coloured successively green, blue, violet, red, and yellow. By placing the platinum terminals of a battery of four Grove's cells in some bile, the succession of colour reactions due to the oxidation of bilirubin will be produced round the anode, and can be observed to great advantage. On reversing the current the colour changes occur in the opposite order.

By the reduction of bilirubin in alkaline solution by means of sodium amalgam, Maly obtained hydrobilirubin, which is apparently a mixture of two substances, one of which mesobilirubinogen ($C_{33}H_{44}N_4O_6$) gives colorless crystals and is identical with urobilinogen of the urine which oxidises in the air to form urobilin. Hydrobilirubin is

also identical with, or closely related to, stercobilin, the colouring matter of fæces in a state of health, but is absent during an attack of jaundice, when the fæces are slate-coloured.

Biliverdin, $C_{33}H_{36}N_4O_8$ (Kuster: *Z. physiol. Chem.*, **121**), exists in human bile, but is especially characteristic of the bile of herbivorous animals. It is uncertain if it is a single substance. It is produced with great facility by the oxidation of bilirubin, from which it differs in colour (dark green), its insolubility in chloroform, and its ready solubility in alcohol and glacial acetic acid. It is also soluble in benzene and in carbon disulphide, but is only slightly soluble in ether. If yellow nitric acid is added to an alcoholic solution of biliverdin a bluish-violet coloration is produced, changing to red and finally to yellow with excess of acid.

Detections of Bile Pigments in Urine.—The colouring matters of bile are not present in normal urine, but in certain diseases (jaundice, etc.) they exist in very appreciable amount. Such urine exhibits a yellowish-green, green, greenish-brown, or almost black colour; bilirubin predominates in bilious urine of a saffron yellow colour; while biliverdin and other oxidation products are present in greenish urine. Bilious urine gives a yellow froth on agitation, and stains linen and filter paper yellow.¹

A variety of tests have been proposed for the detection of bile pigments in urine, but the following are the most delicate, and answer every purpose:

Gmelin's test consists in treating the urine with strong nitric acid and observing the change of colour produced. The reaction is best observed by allowing some of the urine to run gently on to the surface of some yellow nitric acid contained in a test-tube. If bile pigments are present, a green ring will become apparent at the point of contact, while below this will appear violet, red, and yellow zones, in the order named. The green colour alone is characteristic of bilious urine, since indigogens give rise to blue and red colorations. The urine of patients who have taken potassium iodide also gives a red zone with nitric acid. Various modifications of Gmelin's reaction have been proposed.

Hammarsten's Test.—A mixture of 1 volume nitric acid and 19 volumes hydrochloric acid (both acids about 25%) is made, and when

¹ If the dyed filter paper is treated with a drop of nitric acid, the margin of the spot will become violet or deep blue, while the centre gradually changes to emerald green.

it has been allowed to stand until yellow in colour, 1 volume of this acid mixture is added to 4 volumes of alcohol. If a solution containing bile pigments is added to a few c.c. of this colourless solution, a series of colours corresponding to the colours obtained in Gmelin's test is obtained. In the presence of blood or other pigments it is best to place about 10 c.c. of the acid or nearly neutral (not alkaline) fluid in a tube to add barium chloride solution and to centrifuge for a few minutes. The supernatant fluid is decanted, and the sediment treated with 1 c.c. of the above reagent and centrifuged again. A beautiful green solution is obtained, and on adding more of the reagent it becomes blue, violet, red and reddish-yellow. A green colour can be obtained in a urine containing 1 part of pigment in 500,000 to 1,000,000 parts urine. Calcium chloride is used to better advantage in the presence of large quantities of other pigments.

Huppert's Test.—If a solution of alkali bilirubin is treated with milk of lime or with calcium chloride and ammonia, a precipitate is produced consisting of calcium bilirubin. If this moist precipitate, which has been washed with water, is placed in a test-tube and the tube half filled with alcohol which has been acidified with hydrochloric acid, and heated to boiling for some time, the liquid becomes emerald-green or bluish-green in colour. On applying this test for bile pigments in urine it is treated with calcium hydroxide or first with calcium chloride and then with a solution of sodium or ammonium carbonate, and the precipitate collected on a filter and treated as above described.

Rosin's test for bile pigments consists in allowing very dilute iodine solution or bromine water to flow on to the surface of the urine from a pipette. A grass-green ring is produced at the junction of the two strata.

Another reliable test for bile pigments in urine is to treat 30 c.c. (or 1 oz.) of the sample with about one-third of its bulk of a 20% solution of zinc acetate, after previously neutralising most of the free acid by sodium carbonate. The voluminous precipitate is filtered off, washed, and treated with a little ammonia. In presence of bile pigments the ammoniacal liquid is usually fluorescent, and either at once or on standing shows the absorption spectrum of bilicyanin, characterised by bands on each side of the D line, and a third between B and F.

It must be remembered that some urines which contain, when fresh, only a small amount of bile pigment, will, after being exposed to the air for several days, show no bilirubin whatever, urobilin having taken its place.¹ The source of urobilin in the fæces is also doubtless the bile pigment, unaltered bile pigment never occurring in normal fæces.

Urinary urobilin exhibits a green fluorescence when the urine is rendered ammoniacal, and a few drops of zinc chloride are added. It shows a well-marked absorption band between the Fraunhofer lines b and F. The coloured products formed from bilirubin by oxidation, with the exception of the final yellow product choletelin, all exhibit a similar fluorescence with ammonia and zinc chloride, and show an absorption band near F; but it is less sharply defined than in the spectrum of urobilin which, however, they closely resemble.

Bilirubin and its various derivatives are best determined by spectro-photometric methods. In jaundice bile pigment accumulates in the blood serum and its estimation is of diagnostic importance. Separate the serum from 4 or 5 c.c. of freshly drawn un hæmolyzed blood. According to the depth of its color, dilute 1 c.c. with 0.9% NaCl in a graduated cylinder until an approximate match with standard potassium dichromate (a 0.01% solution containing 2 drops of conc. H_2SO_4 per 500 c.c.) is obtained. Compare in a colorimeter. The reading of the standard over the reading of the unknown, times the dilution, gives the icteric index. This is normally 4-6; in latent jaundice 6-15. Above this value icteric symptoms may be observed.

In the Van der Bergh test (*Presse méd.*, 1921, 29, 441) the serum is first treated with a diazotising reagent, comparison being made with a standard solution of ferric thiocyanate in ether. For discussion see Hawk and Bergeim: *Practical Physiological Chemistry* or other books on diagnostic methods. See also Greene et al (*Arch. Int. Med.*, 1925, 36, 248).

The following process for estimating the bile pigments in bile has been suggested by Cзыlhary, Fuchs and v. Furth (*Biochem. Z.*, 49, 120):—5 c.c. of bile are heated with 5 c.c. of 10% sodium hydroxide for 30 minutes on a steam-bath under a reflux condenser; this treatment changing the bilirubin to biliverdin. The solution is mixed

¹ Fresh urine containing but little urobilin often becomes darker on exposure to the air, a change probably due to the formation of urobilin from urobilinogen.

with 30 c.c. of 95% alcohol, and the precipitate formed is removed by filtering. The filtrate is cleared by adding a few drops of hydrochloric acid, and the green solution is compared in a colorimeter with a 0.02% alcoholic solution of Schuchardt's biliverdin.

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THE CYANOGEN COMPOUNDS

BY G. H. BUCHANAN

CYANOGEN AND THE CYANOGEN HALIDES

THE CYANOGEN COMPOUNDS IN GENERAL

The cyanogen compounds are characterised by the presence of the monovalent radical CN. This radical behaves as an element and may be transferred intact from one compound to another. The compound CN itself does not exist in the free state; when liberated, two or more of the radicals combine to form a single molecule. Thus by union of two such radicals the gas cyanogen is formed.

Cyanogen resembles the halogens, particularly chlorine, and its compounds are similar to the corresponding compounds of chlorine. Its hydrogen compound, hydrocyanic acid, reacts with metals to yield salts, and the properties of both acid and salts recall the properties of the corresponding chlorides. Thus the silver salts of hydrocyanic acid and of hydrochloric acid are similar in appearance and in many chemical properties; both are white and curdy in appearance, and both are insoluble in water and in dilute acids but soluble in ammonia.

The most characteristic property of the cyanogen compounds, and that on which most of their important applications are based, is the ease with which they enter into double compounds, a property also exhibited by the halogens, although in less degree. These double compounds may be divided into two classes by their behaviour towards dilute mineral acids. Members of one class are decomposed by the dilute mineral acids, yielding hydrocyanic acid and an insoluble simple cyanide. Members of the second class do not liberate hydrocyanic acid on acidification and must be considered as salts of characteristic acids. The double cyanide of sodium and silver $[\text{NaAg}(\text{CN})_2 \text{ or } \text{NaCN}.\text{AgCN}]$ is representative of the first class, whilst the iron cyanogen compounds, the ferro- and ferricyanides, are typical of the second.

Like chlorine, cyanogen forms an oxy-acid, cyanic acid being analogous to hypochlorous acid of the chlorine system. The oxygen of cyanic acid may be replaced by sulphur, thiocyanic acid resulting. The salts of this latter acid are very stable and have some industrial importance.

By combination of the cyanogen radical with a halogen, a cyanogen halide is formed. Both cyanogen chloride and cyanogen bromide have found a limited technical application.

These cyanogen halides react with ammonia to produce cyanamide. Cyanamide, although thus closely related to the true cyanogen compounds, differs from them in many important respects; it has but little tendency to produce double salts with the heavy metals and it does not enter into complex ions. The calcium salt of cyanamide is a compound of great commercial importance.

The tendency to polymerisation exhibited by many members of the cyanogen group is analogous to their tendency toward formation of double compounds; cyanic and thiocyanic acid, cyanogen and cyanamide all exhibit this property in marked degree.

By substitution of an alkyl group for hydrogen, two series of compounds may be derived from hydrocyanic acid—compounds in which the hydrogen is linked with carbon, the cyanides or nitriles, and compounds in which the hydrogen is linked with nitrogen, the isocyanides, isonitriles or carbylamines. This isomerism is another of the characteristics of the cyanogen compounds.

All cyanide compounds, irrespective of the form in which the cyanogen is combined, will yield all their nitrogen as ammonia when heated to a high temperature with an excess of acid or of soda-lime. All of them yield the whole of their nitrogen as ammonia when digested with concentrated sulphuric acid, as in the Kjeldahl process.

CYANOGEN

Cyanogen may be produced by combination of carbon and nitrogen when nitrogen gas is passed through the carbon arc. For its preparation in the laboratory the easily decomposable cyanides of silver or mercury are usually employed. When these are heated cyanogen is liberated, the residue consisting of free metal together with a small amount of paracyanogen, a polymer of cyanogen. Both

methods of preparation are described by J. W. Terwen (*Z. physik. Chem.*, 1916, **91**, 469). Its preparation from potassium cyanide and cupric sulphate is described by Jacquemin (*Compt. rend.*, 1885, **100**, 1005), and from potassium ferrocyanide and mercuric chloride by Kemp (*J. prakt. Chem.*, 1844, [1], **31**, 63).

The gas is colourless and has a pungent odour. Although poisonous, it is said to be less deadly than hydrocyanic acid (Lewin, *Lehrbuch der Toxicologie*, 2nd Ed., p. 163). It makes defibrinated blood black and destroys the red corpuscles.

The blood of animals poisoned with cyanogen is said to show the same change of the absorption spectrum as dead blood after the absorption of cyanogen.

The formation of the gas from its elements causes an absorption of heat—82,000 calories per grm. molecule according to Berthelot (*Ann. chim. phys.*, 1879, [5], **18**, 347), or 67,400 according to Thomsen (*Ber.*, 1880, **13**, 152). According to Lewis and Randall (*Thermodynamics*, 1923, 592) both values are probably much too low.

The boiling point of cyanogen is given by Chappius (Bakhuys Roozeboom, *Die heterogenen Gleichgewichte*, 1901, **I**, 176) as -20.7° . Terwen's (*loc. cit.*) latest value is -21.35° . The earlier freezing point of Faraday, $-34^{\circ}.4$, differs considerably from Terwen's recent value, $-27^{\circ}.92$. Figures recently published by Perry and Bardwell (*J. Amer. Chem. Soc.*, 1925, **47**, 2629), are in close agreement with the data of Terwen. The critical constants of cyanogen, according to three authorities, are as follows:

Author	Reference	Critical temp.	Critical pressure
Dewar.....	<i>Phil. Mag.</i> , 1884, [v], 18 , 210 <i>Jahresberichte</i> , 1885, p. 60	124°	61.7 atmospheres
Cardoso & Baume...	<i>J. Chim. Phys.</i> , 1912, 10 , 512	128.3	59.8 atmospheres
Terwen.....	<i>loc. cit.</i>	126.6	58.2 atmospheres

Terwen gives the following values for the vapour pressure of liquid cyanogen:

Temp.	Vapour pressure	Temp.	Vapour pressure
-71.6°	1.8 cm. Hg.	49.5°	11.46 atmospheres
-21.35	1.00 atmosph.	75.5°	21.4 atmospheres
0.0°	2.42 atmosph.	87.7°	28.14 atmospheres
21.15°	5.07 atmosph.	126.55°	58.2 atmospheres
		(critical temp.)	

These values differ considerably from the earlier values of Faraday (*Ann.*, 1845, **56**, 158) and Bunsen (*Ann. Physik.*, 1839, **46**, 101).

The solubility of cyanogen in water at 20° has been reported by Gay Lussac as 4.5 volumes. According to Naumann (*Z. Elektrochem.*, 1910, **16**, 772) no constant solubility can be obtained at this temperature on account of hydrolysis. At 0° Naumann found hydrolysis to be negligible and the solubility to be approximately 5 volumes.

Pure dry cyanogen may be kept indefinitely in closed tubes, but it becomes unstable in presence of water (Schutzenberger, *Bull. Soc. Chim.*, 1885, [ii], **43**, 306). Decomposition is indicated by the formation of brown decomposition products. The stability of cyanogen solutions may be increased by acidification (Zettel, *Monatsh.*, 1893, **14**, 223).

The reactions of cyanogen recall those of free chlorine. It combines with alkali metals with violence to form the corresponding cyanides. With aqueous solutions of the alkalies it reacts to form salts of hydrocyanic and cyanic acids. This reaction with alkaline solutions is one of the most characteristic reactions of cyanogen and is employed for its identification and estimation. With aqueous solutions of hydrochloric acid cyanogen yields oxamide.

Paracyanogen.—According to A. Smits ("The Theory of Allotropy," 1922, pp. 225, 270) paracyanogen bears the same relation to cyanogen that red phosphorus bears to yellow phosphorus. It is formed, together with cyanogen, by heating silver cyanide; it may be produced from cyanogen by heat. (Briner and Wroczynski, *Compt. rend.*, 1910, **151**, 314.)

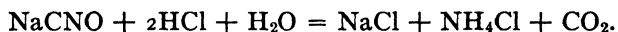
It is a brown-black amorphous body, insoluble in water and alcohol, soluble in concentrated sulphuric acid. With potassium or sodium hydroxide it forms the corresponding alkali cyanate.

Detection and Determination of Free Cyanogen.—Cyanogen is absorbed by solutions of caustic alkalies according to the reaction—



Since free cyanogen rarely occurs unassociated with hydrocyanic acid, the detection of cyanide ion in the absorbing solution is not evidence of the presence of free cyanogen in the original gas mixture. Detection of cyanate, however, confirms the presence of cyanogen. This is usually accomplished by acidification and boiling to remove

hydrocyanic acid. Cyanate is quantitatively converted into ammonia by the acidification, and the ammonia thus formed may be detected or determined by usual methods. The reaction is—



Wallis (*Ann.*, 1905, **345**, 353) called attention to the fact that hydrocyanic acid is quantitatively absorbed by acid silver nitrate, whilst cyanogen is not acted upon. Rhodes (*J. Ind. Eng. Chem.*, 1912, **4**, 652) studied this separation and defined the analytical conditions. His recommendations for the identification and estimation of free cyanogen in presence of hydrocyanic acid are as follows: For qualitative tests the gases are passed through two scrubbers (test tubes with side necks) the first containing 10 c.c. of a 10% solution of silver nitrate acidified with 1 drop of *N*/6 nitric acid and the second 10 c.c. of *N*/2 caustic potash. A slow current of air is then passed through the scrubbers for ten minutes to displace dissolved cyanogen from the silver nitrate solution. 5 c.c. of 10% ferrous sulphate solution and 1 drop of ferric chloride solution are now added to the solution from the caustic scrubber, followed, after about 15 minutes, by sufficient dilute sulphuric acid to dissolve the precipitated ferrous and ferric hydroxides. A green coloration or a blue precipitate identifies cyanogen in the original gas sample. By this method 0.3 c.c. of cyanogen can be detected in 10 c.c. of hydrocyanic acid.

For the determination of free cyanogen four scrubbers are recommended, the first two each containing 5 c.c. of standard (*N*/10) silver nitrate with one drop of dilute nitric acid. The third tube contains 10 c.c. of approximately *N*/2 caustic potash free from chloride, while the fourth contains 5 c.c. of the same solution. After passage of the gas sample and aeration for 30 minutes the contents of the two caustic scrubbers are transferred to a beaker and a known volume of standard silver nitrate added, the silver addition being in excess of the amount required completely to precipitate the cyanide in the solution. After acidification with dilute nitric acid to dissolve silver oxide the mixture is filtered, the precipitate washed, and the excess of silver determined in the filtrate and washings by the Volhard method. The hydrocyanic acid absorbed by the acid silver nitrate scrubbers may be determined, if desired, by filtering off the precipitated silver cyanide and titrating the excess silver in solution.

CYANOGEN HALIDES

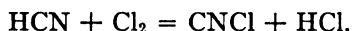
Cyanogen Chloride.—Recent investigations in connection with the production of toxic gases for war uses have given considerable attention to cyanogen chloride on account of its poisonous and irritant properties. A review of the earlier literature relating to the gas may be found in an article by Jennings and Scott (*J. Amer. Chem. Soc.*, 1919, 41, 1241).

Three methods which have given most satisfactory results for the preparation of cyanogen chloride are as follows:—

(a) *Method of Langlois* (*Ann. chim. phys.*, 1861, [iii], 61, 481).—The gas is prepared by the action of chlorine on an alkali cyanide. As modified by Jennings and Scott (*loc. cit.*), the sodium cyanide is slightly moistened with water, carbon tetrachloride is added to serve as a diluent, and chlorine gas is passed into the mixture until absorption is complete. This method gives good results in small scale operations, but is difficult to conduct in large scale work on account of the large amount of heat liberated during the chlorination. (Price and Green, *J. Soc. Chem. Ind.*, 1920, 39, 98.)

(b) *Method of Held* (*Bull. Soc. Chim.*, 1897, [iii], 17, 287). The double cyanide of zinc and potassium, $K_2Zn(CN)_4$, is treated with chlorine. According to Price and Green this method gives only fair results.

(c) *Method of Berthollet* (*Ann. chim. phys.*, 1790, [i], 1, 36).—Chlorine is bubbled into a dilute solution of hydrocyanic acid, the mixture being strongly cooled during chlorination. At the end of the chlorination the temperature is allowed to rise and the cyanogen chloride is distilled off. The reaction is,—



This method was selected by Price and Green as most satisfactory for large scale work. It has also been approved by Sernagiotto (*Giorn. Chim. Ind. Appl.*, 1921, 3, 153).

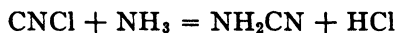
Cyanogen chloride is a highly poisonous gas with pronounced lachrymatory properties. Kohn-Abrest (*Ann. Fals.*, 1915, 8, 215) classifies it as an irritant poison. According to Reed (*J. Ind. Hygiene*, 1920, 2, 140) the symptoms of cyanogen chloride poisoning recall those of hydrocyanic acid, but are supplemented by certain additional symptoms which may be due to chlorine. While severe symptoms produced by a single large dose of the simple cyanides

may not be followed by chronic symptoms, a single large dose of cyanogen chloride may cause death as a result of chronic symptoms.

The more important physical properties of cyanogen chloride are as follows:

Author	Reference	Melting point, °C.	Boiling point, °C.	Density
Regnault,	(<i>Jahresber.</i> , 1863, 70, 74)	-7.4°	+12.66°
Price & Green, . . .	<i>loc. cit.</i>	-8°	+13° uncorrected	1.218 at 4°/4°
Jennings & Scott, . .	<i>loc. cit.</i>	-5° to -6°	+13° at 748 mm.
Nauguin & Simon	(<i>Ann. Chim.</i> , 1921, 15, 18)	-6.5°	+12.5°	1.222 at 0°

Cyanogen chloride is moderately soluble in water, readily soluble in alcohol and in ether. In aqueous solution it is slowly hydrolysed with formation of ammonium chloride and carbon dioxide. In acid solution hydrolysis increases with strength of acid, being complete in a few hours with 9*N* hydrochloric acid (Price and Green). It is completely decomposed by caustic alkalies, with formation of salts of hydrochloric and cyanic acids. The reaction of cyanogen chloride with ammonia in ethereal solution is of theoretical importance; cyanamide is formed according to the equation.—



(Cloeze and Cannizzaro. *Compt. rend.*, 1851, 32, 62).

The tendency towards polymerisation which is so highly developed in the cyanogen compounds is exhibited by cyanogen chloride in unusual degree. In presence of mineral acids, particularly in the absence of water, polymerisation occurs, with formation of cyanuric chloride. This reaction is rapid in presence of large amounts of acid, but probably never occurs with explosive violence (Jennings and Scott). Perfectly pure cyanogen chloride, according to Price and Green, may be kept for months without polymerisation, even when exposed to direct sunlight.

The Public Health Service of the United States recommends the fumigation of ships, barracks and other infested structures by the combined action of cyanogen chloride and hydrocyanic acid. The method is fully described in Public Health Reports 1922, 37, 2744. Sodium cyanide and sodium chlorate are mixed dry and are then dropped into a vessel containing dilute hydrochloric acid. It is

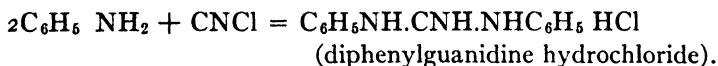
claimed for the mixture of gases thus produced that the lachrymatory effect of the cyanogen chloride is so intense that human beings are expelled from the danger area before the concentration of poisonous gases has become lethal, thus minimising the danger of fatal accidents during the fumigation operation. The mixed gases are said to be highly toxic toward rodents, bats and insect pests, while they have no injurious action on foods, fabrics, leather or metals, with the possible exception of nickel.

The British Ministry of Health has also issued a Memorandum on the fumigation of ships with hydrogen cyanide (H. M. Stationery Office, 1928).

To reduce the fire risk, and moderate the rate of reaction, talc may be mixed with the sodium chlorate. The fire hazard may also be reduced by use of a mixture of liquid hydrocyanic acid and liquid cyanogen chloride which is now an article of commerce.

According to Meifert and Garrison (*U. S. Dept. of Agriculture, Bull.*, 893, (1920, 1-16) cyanogen chloride may not be substituted for hydrocyanic acid in the fumigation of greenhouses on account of its injurious action on the plants.

Cyanogen chloride is also used in large amount in the United States in the manufacture of the di-substituted guanidines, diphenylguanidine and ditolylguanidine, two important rubber accelerators. The reactions are as follows:



Determination.—A determination occasionally required is that of cyanogen chloride in admixture with hydrocyanic acid. If a gaseous mixture is to be examined, the gases may be absorbed in dilute caustic soda solution, the hydrocyanic acid forming sodium cyanide and the cyanogen chloride forming sodium cyanate and sodium chloride. The mixture is then acidified and the hydrocyanic acid boiled off and, if desired, absorbed in caustic soda and titrated as on page 504. Sodium cyanate is hydrolysed by this treatment, yielding an ammonium salt which may be distilled off and absorbed in standard acid and thus determined. If a liquid mixture of cyanogen chloride and hydrocyanic acid is to be examined it should be distilled at low temperature and the vapours absorbed in dilute caustic soda solution. It is not permissible to neutralise the liquid

without distilling, since it may contain cyanuric chloride or cyanuric acid, both resulting from polymerisation of cyanogen chloride.

Cyanogen chloride may also be determined iodometrically as described under cyanogen bromide.

Cyanuric Chloride, $(\text{CN})_3\text{Cl}_3$, melts at 146° and boils at 190° . Like cyanogen chloride, it causes intense lachrymation. It is slightly soluble in water, its aqueous solutions slowly decomposing in the cold with formation of hydrochloric and cyanuric acids. Cyanuric chloride may also be prepared by heating potassium thiocyanate with chlorine, or by the action of phosphorus pentachloride on dry cyanuric acid.

Cyanogen Bromide.—Cyanogen bromide may be produced in the laboratory by the methods described for the production of the chloride. The pure compound melts at 52° and boils at 61.3° under 750 mm. pressure. Its vapour pressure, according to Baxter (*J. Amer. Chem. Soc.*, 1920, **42**, 1386), is 119.5 mm. at 25° , and 21.2 mm. at 0° . Its behaviour towards reagents and solvents recalls the behaviour of the chloride. It polymerises to cyanuric bromide, a white powder with a melting point above 300° .

Cyanogen bromide had at one time a considerable industrial importance in connection with the extraction of gold, but the process employing it, that of Sulman and Teed, is now chiefly of historic interest. Although cyanogen bromide itself is without solvent action on the precious metals, it has, when used in conjunction with an alkali cyanide, the effect of greatly increasing the rate of the oxidising action so essential for precious metal extraction. With the development of improved methods of cyanidation, particularly with respect to finer grinding and better roasting, the need for these special solvents has largely disappeared. The process is rarely used at the present time, its only application being on the telluride ores of the Kalgoorlie district in Australia and in the Kirkland Lake district in Ontario.

The "bromcyanogen" is prepared as needed. To a dilute solution of sulphuric acid is added the "bromine salts," a mixture of sodium bromate and sodium bromide. Sodium cyanide solution is now added to the bromine solution until practically all of the bromine has been consumed. The stock solutions of cyanogen bromide are tested by adding potassium iodide and titrating the liberated iodine with sodium thiosulphate. In examining such solutions it is impor-

tant that any undecomposed bromate or free acid should be removed before proceeding with the analysis. This is accomplished by neutralising with sodium carbonate and afterward acidifying with acetic acid before adding the potassium iodide. A description of modern metallurgical practice with cyanogen bromide, together with a bibliography, may be found in an article by Stevens and Blackett (*Trans. Inst. Mining and Metall.*, 1919-1920, 29, 280).

Cyanogen Iodide.—Cyanogen iodide is produced by the action of iodine on a solution of an alkali cyanide. When the action is complete the mixture is heated and cyanogen iodide distilled off. The solid volatilises slowly at ordinary temperature. Commercial iodine sometimes contains cyanogen iodide, which may be detected by the following method (Meineke, *Z. anorg. Chem.*, 1892, 2, 157-168): A solution of sodium thiosulphate is added to the iodine solution, after acidification with a drop of HCl, until nearly all the iodine has reacted. The solution is now tested for cyanide by the Prussian blue test as described on page 493.

HYDROCYANIC ACID AND THE SIMPLE CYANIDES

Hydrocyanic Acid.—The simple metallic cyanides are salts of hydrocyanic acid, also known as prussic acid. According to Franklin (*J. Phys. Chem.*, 1923, 27, 167) the familiar reactions of the acid and its salts may be explained, and new relations predicted, by viewing it in three ways.

1. As an ammonocarbonous acid, or carbylamine, having the structural formula $\text{H}-\text{N}=\text{C}$. The metallic cyanides are to be considered salts of this acid.

2. As an ammono formaldehyde $\text{H}-\text{C}\equiv\text{N}$.

3. As a formic anammonide or nitrile $\text{H}-\text{C}\equiv\text{N}$.

Free hydrocyanic acid is considered to have the formula $\text{H}-\text{C}\equiv\text{N}$. (Usherwood, *J. Chem. Soc.*, 1922, 121, 1604; see also Enklaar below.) In its ready polymerisation, in its condensation to aldol-like bodies and in its reaction with acid sulphites to form addition compounds, the free acid exhibits its analogy to formaldehyde; while in its reactions with water, forming first formamide, which, according to the Franklin system, is a mixed aquo-ammono-formic acid, and finally aquo or ordinary formic acid, it exhibits the properties of formic anammonide or formonitrile.

In a series of papers on the tautomerism of hydrocyanic acid Enklaar (*Rec. trav. Chim. Pays Bas*, 1923, **42**, 1,000; 1925, **44**, 889; 1926, **45**, 414) has described a method for the identification of the two modifications of hydrocyanic acid. These are said to give mercury methyl compounds of different melting points; mercury methyl cyanide has a melting point of about 90° , and mercury methyl isocyanide of about 58° .

The alkali and the alkaline earth cyanides, as well as the cyanide of mercury, are soluble in water. The salts of the heavy metals are insoluble. The general properties of these compounds recall the properties of the corresponding halogen salts.

Formation and Occurrence in Nature.—Hydrocyanic acid may be formed directly from its elements in the electric arc; better yields are secured when the carbon and hydrogen are supplied as a hydrocarbon. Mixtures of less than two per cent. by volume of acetylene in nitrogen are converted quantitatively (Koenig and Hubbuch, *Z. Elektrochem.*, 1922, **28**, 202). It is also produced when ammonia and carbon monoxide are conducted over a catalyst at temperatures between 400° and 500° . Methylamine yields hydrocyanic acid and hydrogen when heated to temperature approximating 1000° ; this reaction is utilised commercially in the production of cyanides by the destructive distillation of the schlempe or beet-sugar refuse.

Hydrocyanic acid occurs in nature in combination as cyanogenetic glucosides. These compounds liberate hydrocyanic acid, after the death of the plant, by the hydrolytic action of an enzyme. The cherry laurel is a familiar cyanogen-containing plant, having about 0.2% hydrocyanic acid. Rosenthaler (*Schweiz. Apoth. Ztg.*, 1921, **59**, 10, 22) has studied the distribution of the glucoside in various parts of the plant at different seasons. He finds that the hydrocyanic acid content of the plant decreases as the season progresses, and he makes the interesting observation that each successive leaf on a particular branch shows a different hydrocyanic acid content, the amount increasing from an average of 0.14% at the bottom to 0.45% at the top.

Other plants containing substantial amounts of hydrocyanic acid are bitter almonds (0.15% HCN), wild cherry (0.03%), linseed (0.03%) and rangoon beans (0.025%). Sudan grass sometimes causes poisoning of cattle in South Africa, and the same has been

observed of ordinary sorghum. Small amounts of hydrocyanic acid may be found in a very great number of plants; indeed, its occurrence is so general that it has been considered to be one of the intermediate products in the synthesis of proteins by the plant. Treub's hypothesis (*Annales du Jard. botan. de Buitenzorg.*, 1896, 13, 1) considers it to be the first recognised product of nitrogen assimilation. According to this theory it should be possible to detect it, at least in traces, in all plants. Rosenthaler (*Biochem. Z.*, 1922, 134, 215) has attempted to confirm Treub's hypothesis by examining 88 typical plants by a particularly sensitive test. Traces of hydrocyanic acid were detected in 56 of the 88 plants examined.

Lehmann and Gundermann (*Arch. Hyg.*, 1912, 76, 98) have identified hydrocyanic acid in the smoke of tobacco. The amount varies with different brands of cigars from 0.02% to 0.04%. Thus the total hydrocyanic acid obtained from the smoking of a strong cigar is 2 to 4 mg. The hydrocyanic acid content of the smoke produced in average smoking is about 0.1 mg. per litre.

Amygdalin, the glucoside of hydrocyanic acid, is decomposed by the enzyme which is nearly always found associated with it, or by boiling with dilute acids, yielding hydrocyanic acid, benzaldehyde and glucose.

The hydrocyanic acid content of certain medicinal preparations is derived from amygdalin. Examples of such preparations are bitter almond oil, containing 2 to 4% HCN, and cherry laurel water, with 0.17% HCN.

Manufacture.—The hydrocyanic acid of commerce is produced almost exclusively by the action of sulphuric acid on sodium or calcium cyanide. Pelton and Swartz (*Chem. Met. Eng.*, 1919, 20, 165) describe an experimental installation for the production of hydrocyanic acid from sodium cyanide on a semi-commercial scale. They report a 78% yield from sodium cyanide to liquid hydrocyanic of 90–95% purity. Commercial installations operating on a large scale obtain a considerably higher yield than this and produce an acid containing not less than 97% actual hydrocyanic acid.

Properties.—The latest study of the physical properties of hydrocyanic acid is by Perry and Porter (*J. Amer. Chem. Soc.*, 1926, 48, 299). The vapour pressures of solid and of liquid hydrocyanic acid are shown in the following table:

Temp. ° C.	Pressure Mm. (corr.)		$P_0 - P_c$
	Observed (P_0)	Calculated (P_c)	
SOLID			
- 29.40	50.24	48.66	+1.58
- 19.70	95.49	95.49	0.00
- 15.15	128.75	128.75	0.00
LIQUID			
- 8.02	183.08	183.26	-0.18
0.00	264.39	265.42	-1.03
3.01	303.71	303.42	+0.29
6.83	359.11	357.84	+1.27
12.20	448.93	448.20	0.73
16.67	538.35	537.14	1.21
21.43	647.87	647.17	0.70
24.57	729.04	729.65	-0.61
25.57	757.27	757.78	-0.51
27.32	807.88	808.90	-1.02

The following equation is derived for the vapour pressure of the solid

$$\log_{10} P_{\text{mm.}} = 9.33902 - (1864.8/T)$$

and for the liquid

$$\log_{10} P_{\text{mm.}} = 7.744603 - (1453.063/T).$$

The latent heat of vaporisation and sublimation were calculated by means of the approximate Clapeyron equation to be 246.84 cal. per gram. at the normal point and 316.13 cal. per gram. for the solid. The triple point was found to be -14.86 at 131.16 mm. By subtracting the latent heat of vaporisation of liquid hydrocyanic acid at the triple point from the latent heat of sublimation the value 69.29 cal. per gram. was obtained for the latent heat of fusion at the triple point.

Owing to its high heat of vaporisation, the temperature of liquid hydrocyanic acid is rapidly lowered by spontaneous evaporation; it may be frozen to a solid by passing a current of air over its surface.

In the manufacture and sale of liquid hydrocyanic acid, its specific gravity is employed as a measure of the purity of the acid. This industry has reached its highest development in Southern California, where the specific gravity tables developed by Gray and Hulbirt (Bull. 308, University of California Experiment Station, 1919) are recognised as official. These tables are given in an abbreviated form on page 483.

A later study by Walker and Marvin (*Ind. Eng. Chem.*, 1926, 18, 139) yielded slightly different, and probably more accurate, values. Their tables are reproduced in full on page 486 and 487.

Both of the above tables apply to the concentrated hydrocyanic acid solutions used in commercial fumigation. Shirado (*Bull. Chem. Soc. Japan*, 1927, 2, 122) gives specific gravity data over a wider range as follows:

SPECIFIC GRAVITY OF AQUEOUS HCN AT 18.0° C.

Concentration (% wt.)	Specific gravity $\frac{18^\circ}{18^\circ}$
5.05	0.9927
9.77	0.9841
10.04	0.9838
14.58	0.9732
19.70	0.9593
20.29	0.9578
26.90	0.9376
33.01	0.9167
39.26	0.8987
46.01	0.8757
47.68	0.8658
52.00	0.8518
55.37	0.8428
60.23	0.8290
60.76	0.8270
69.50	0.7953
70.93	0.7925
79.54	0.7598
80.18	0.7582
88.55	0.7289
89.64	0.7265
100.00	0.6919

THE SPECIFIC GRAVITY OF HYDROCYANIC ACID AND WATER
MIXTURES FOR USE WITH SPECIFIC GRAVITY HYDROMETER
CALIBRATED AT 15° C.
(Gray and Hulbirt)

Observed specific gravity	Observed temperature			
	5°	10°	15°	20°
	Corresponding percentage of hydrocyanic acid			
0.692	99.9
0.694	99.3
0.696	98.7
0.698	100.0	98.1
0.700	99.6	97.5
0.702	98.9	96.9
0.704	98.4	96.3
0.706	99.8	97.8	95.7
0.708	99.2	97.1	95.1
0.710	98.6	96.5	94.5
0.712	98.0	95.9	93.9
0.714	99.6	97.4	95.3	93.4
0.716	99.0	96.7	94.7	92.8
0.718	98.4	96.1	94.1	92.2
0.720	97.8	95.5	93.6	91.6
0.722	97.1	94.9	93.0	91.0
0.724	96.5	94.3	92.4	90.4
0.726	95.9	93.7	91.8	89.8
0.728	95.3	93.1	91.2	89.3
0.730	94.7	92.5	90.6	88.7
0.732	94.0	91.9	90.0	88.1
0.734	93.4	91.3	89.4	87.5
0.736	92.8	90.7	88.8	87.0
0.738	92.2	90.1	88.2	86.4
0.740	91.6	89.5	87.6	85.8
0.742	91.0	88.9	87.0	85.2
0.744	90.4	88.3	86.4	84.6
0.746	89.8	87.7	85.8	84.1
0.748	89.1	87.1	85.2	83.5
0.750	88.5	86.5	84.6	82.9
0.752	87.9	85.9	84.0	82.4
0.754	87.3	85.3	83.5	81.8
0.756	86.7	84.7	82.9	81.2

Hydrocyanic acid is miscible in all proportions with water, alcohol, ether and chloroform. A contraction in volume and a lowering of temperature result when the acid is mixed with water. Hydrates are believed to be formed, but none have been isolated. The boiling points of dilute solutions of hydrocyanic acid show a sharp deflection at a composition of 18% hydrocyanic acid, boiling

point 39° C. This composition corresponds to a hypothetical hydrate with the formula $\text{HCN}, 7\text{H}_2\text{O}$.

The dissociation constant of hydrocyanic acid at 18° is 13.2×10^{-10} (Walker and Cormack, *J. Chem. Soc.*, 1900, 77, 16). It is thus one of the weakest of the acids. It reacts with chlorine to produce cyanogen chloride, with hydrogen in presence of a catalyst to produce methylamine, and with water to form a variety of products whose nature and proportions are largely determined by the hydrogen ion concentration of the mixture.

The stability of liquid hydrocyanic acid is a matter of considerable importance to manufacturers and users. In presence of alkalis it rapidly turns brown, ultimately yielding flocks of a brown decomposition-product known as azulmic acid. This transformation results in the evolution of heat and has been known to cause rupture of the container.

Liquid hydrocyanic acid has been shipped in the United States without difficulty for a number of years, and the evidence is to the effect that the hazard in its transportation is not greater than for other liquefied toxic gases. The experience of one large manufacturer of the liquefied acid may be summarised as follows:

1. Commercial hydrocyanic acid should be acid to methyl orange. If alkaline to methyl red indicator (pH greater than 5), it will be unstable. Decomposition may be slow at first, but is catalysed by the products of the reaction.

2. Decomposition is attended by a colour change. The liquid may be stored indefinitely so long as it remains colourless. Decomposition is shown by the appearance of a yellow colouring substance which at first dissolves in the medium and which later separates as brown insoluble flocks. Hydrocyanic acid of yellow colour is to be viewed with suspicion; if brown and opaque, it may be dangerous.

3. Water takes part in the decomposition reactions, and hydrocyanic acid for shipment should not contain more than 3% of water. In such material the decomposition reactions are slow, even when the liquid is alkaline.

4. Liquid of less than 90% concentration will decompose rapidly if of alkaline reaction. Decomposition is attended with heat evolution and proceeds with great rapidity, being auto-catalysed by the

ammonia which is one of the products of the decomposition. Pressures sufficient to rupture the container may be built up.

5. These excessive pressures are due to the vapour pressure of hydrocyanic acid or of ammonium cyanide at the temperature of the reaction. The reaction of decomposition may be represented by the equation:



6. The solid is a black material resembling coke and containing about 45% nitrogen. When heated to a red heat the solid liberates ammonia, water, and hydrocyanic acid.

7. In case of an emergency, containers whose contents are undergoing decomposition may be saved by rapid cooling, for example, by hosing.

The conclusions of Walker and Eldred (*Ind. Eng. Chem.*, 1925, **17**, 1074) are not in entire agreement with the above statements. They call attention to the action of ammonia, caustic soda, sodium cyanide and water in catalysing the polymerisation of hydrocyanic acid. Sulphuric acid and metallic copper are classified as stabilisers. They conclude that rupture of the container is due to the formation of gases, caused by the exothermic polymerisation and decomposition of the liquid.

Wohler and Roth (*Chem. Ztg.*, 1926, **50**, 761, 781) have investigated the explosive properties of hydrocyanic acid. They find that hydrocyanic acid is an explosive in accordance with its energy content. Explosion, however, is induced only by the use of strong detonating substances. Such explosion is favoured by preliminary polymerisation with alkali. The explosive range for hydrocyanic acid and air mixtures is between 12.75 and 27% hydrocyanic acid (*Jahresber. IV, Chem. Tech. Reichsanstalt*, 1924/1925, p. 52).

Commercial Application; Fumigation.—Although hydrocyanic acid finds use in synthetic organic chemistry and in medicine, its principal use is for fumigation. Next to the mining industry the fumigation industry is the largest consumer of alkali and alkaline earth cyanides, used almost exclusively for the production of hydrocyanic acid.

Hydrocyanic acid is extremely toxic to all breathing insects, as well as to rodents and the higher forms of animal life. It is reported also to destroy insects in the egg and larval states (Liston

715	100.000	602.2	98.808	3	97.007	5	97.006	606.2	05.705	3	94.094	504.1	63.703	3	92.092	5	92.101	7	01.390	9	90.5
716	99.700	308.9	98.408	6	97.697	1	96.706	305.9	05.404	7	94.004	203.8	93.403	7	92.602	2	91.801	4	01.090	6	90.3
717	99.400	608.6	98.107	7	97.306	8	96.406	605.6	05.105	6	94.003	603.2	93.102	7	92.301	9	91.501	1	01.790	3	90.0
718	100.000	5	97.807	4	97.006	5	96.105	795.3	04.804	4	94.003	603.2	92.802	1	92.001	6	91.200	8	00.400	89.7	
719	06.992	98.858	407.9	97.507	1	96.706	2	95.805	495.0	04.594	1	93.793	302.9	92.502	1	91.701	3	90.900	5	00.189	8	
720	90.308	98.508	107.6	97.206	8	96.495	0	95.505	104.7	04.293	8	93.493	002.6	92.201	8	91.401	0	90.600	2	89.889	5	
721	90.008	98.207	707.3	96.006	5	95.705	3	94.904	594.0	03.602	2	92.802	402.0	91.601	2	90.800	4	90.080	6	89.288	0	
722	98.408	97.507	196.7	96.305	0	95.403	0	94.603	293.7	03.302	0	92.502	101.7	91.300	0	90.500	1	89.780	3	88.986	8	
723	98.197	97.200	890.4	96.005	6	95.104	7	94.303	603.4	03.002	6	92.201	801.4	91.000	6	90.200	8	89.480	0	88.788	3	
724	97.897	96.900	590.8	95.705	2	94.904	4	94.103	603.1	02.702	3	91.901	501.1	90.700	3	90.000	5	89.288	7	88.587	7	
725	97.497	96.600	295.8	95.404	6	94.604	4	93.803	292.8	02.402	0	91.601	200.8	90.400	0	89.600	2	88.888	4	88.187	7	
726	97.100	96.305	595.5	95.104	6	94.304	8	93.503	492.5	02.101	7	91.300	000.5	90.100	7	89.388	0	88.687	1	87.987	1	
727	96.806	96.005	695.2	94.804	3	93.903	5	93.102	391.2	01.801	4	91.000	600.0	89.800	4	89.088	6	88.387	8	87.687	8	
728	96.506	95.705	394.9	94.504	0	93.603	2	92.702	391.9	01.501	1	90.700	389.9	89.589	1	88.788	3	87.987	6	87.286	8	
729	96.205	95.405	094.6	94.203	7	93.302	9	92.402	091.6	01.200	8	90.400	089.6	89.288	8	88.488	0	87.687	3	86.986	6	
730	95.905	95.104	704.2	93.903	4	93.002	6	92.101	701.3	00.900	5	90.800	789.3	89.688	5	88.887	7	87.987	0	86.986	3	
731	95.605	94.804	303.9	93.593	1	92.702	3	91.801	491.0	00.600	2	89.800	439.0	88.688	2	87.887	0	87.186	7	86.485	0	
732	95.304	94.504	003.6	93.202	8	92.401	9	91.501	190.7	00.389	9	89.500	338.7	88.387	9	87.587	1	86.886	4	86.185	7	
733	95.004	94.123	703.3	92.902	5	92.101	6	91.200	600.4	00.089	6	89.200	538.1	88.087	6	87.286	8	86.586	1	85.885	4	
734	94.694	93.823	403.0	92.602	2	91.801	3	90.900	590.1	89.789	3	88.900	338.1	87.737	3	86.936	6	86.235	8	85.534	8	
735	94.393	93.523	102.7	92.301	9	91.501	0	90.600	259.8	89.490	0	88.638	237.8	87.437	0	86.686	3	85.985	5	85.284	8	
736	94.093	93.222	802.4	92.001	6	91.200	7	90.300	089.5	89.188	7	88.337	987.5	87.136	7	86.386	0	85.685	2	84.984	5	
737	93.793	92.922	502.1	91.701	2	90.800	4	90.000	689.2	88.888	4	88.037	687.2	86.836	4	86.085	7	85.384	9	84.683	2	
738	93.493	92.622	201.8	91.400	9	90.500	1	89.789	388.9	88.588	1	87.887	386.9	86.586	1	85.785	4	85.084	6	84.383	0	
739	93.192	92.321	901.5	91.100	6	90.280	8	89.489	088.6	88.287	8	87.487	086.6	86.285	8	85.485	1	84.784	3	84.083	7	
740	92.892	92.021	601.2	90.800	3	89.980	5	89.188	788.3	87.987	4	87.186	786.3	85.985	5	85.184	8	84.483	1	83.782	4	
741	92.592	91.721	300.9	90.500	0	89.680	2	88.888	488.0	87.687	1	86.886	486.0	85.685	2	84.884	5	84.183	8	83.482	1	
742	92.291	91.420	000.5	90.200	7	89.389	0	88.588	187.6	87.386	8	86.585	185.7	85.384	9	84.583	2	83.882	5	83.181	8	
743	91.991	91.120	300.6	89.900	6	89.080	6	88.287	837.3	87.086	5	86.285	835.4	85.084	6	84.283	9	83.582	2	82.881	5	
744	91.691	90.820	300.8	89.600	3	88.789	3	87.987	437.0	86.786	2	85.985	585.1	84.784	3	83.983	6	83.282	9	82.581	2	
745	91.391	90.520	000.6	89.300	8	88.488	0	87.687	186.7	86.486	0	85.685	284.8	84.484	0	83.683	3	82.982	6	82.281	9	
746	91.090	90.400	089.6	89.088	5	88.388	4	87.688	888.4	86.985	6	86.184	984.5	84.983	7	84.182	0	83.481	3	82.780	6	
747	90.900	90.180	700.3	89.088	1	88.187	7	87.386	886.4	86.985	6	86.184	984.5	84.983	7	84.182	0	83.481	3	82.780	6	
748	90.600	89.880	480.0	88.788	2	87.887	3	87.086	586.1	85.985	0	85.184	684.2	83.983	1	83.182	4	82.481	7	81.780	1	
749	90.300	89.580	188.7	88.487	9	87.587	0	86.686	285.8	85.485	0	84.684	383.9	83.583	1	82.882	4	82.181	7	81.480	0	
750	90.000	89.288	888.4	88.187	6	87.286	7	86.385	085.5	85.184	7	84.384	083.6	83.283	8	82.582	1	81.781	4	81.080	8	

and Gore, *Proc. Asiatic Soc.*, Bengal, 1919, **15**, (ccix). It is not a germicide except at very high concentrations. The gas employed for fumigation may be generated as needed by the addition of sulphuric acid to an alkali cyanide, the so-called pot method, or it may be produced by atomisation and vaporisation of liquid hydrocyanic acid into the space to be fumigated. The former and older method still finds extensive use in sanitary fumigation; it has been largely displaced by the "Liquid process" in the more highly developed industry of horticultural fumigation.

In sanitary fumigation the standard practice is to use one ounce of sodium cyanide (pot method) or one-half ounce of liquid hydrocyanic acid per 100 cubic feet of space for difficult work, and the same quantities for 200 cubic feet in ordinary cases. The higher concentration is recommended for the eradication of vermin, such as lice and bed bugs.

In horticultural fumigation, as exemplified in the citrus-growing areas of California, canvas tents are thrown over the trees, the cubical capacity of the tree is measured by marks on the tents, and the quantity of hydrocyanic acid corresponding to this capacity, as indicated by the dosage schedule, is atomised under the tree by means of an applicator or pump especially designed for the service. The dosage schedule has been worked out empirically by investigations conducted in the State Horticultural Experiment Station. The average dosage corresponds to about one-half ounce of liquid hydrocyanic acid per hundred cubic feet of tent space. Hydrocyanic acid showing from 96 to 98% HCN on analysis is used in this work.

Fumigation of citrus trees with hydrocyanic acid was introduced into California by Coquillett in 1886, the pot method being used. Fumigation with the liquid was first conducted on a commercial scale in 1917, and by 1920 it had almost entirely displaced the earlier method. For a detailed account of California fumigation practice the reader is referred to Woglum and McDonnell, *Bulletin 90 Bureau of Entomology* (1912); R. S. Woglum "Fumigation of Citrus Plants with Hydrocyanic Acid." *Bulletin 907 U. S. Dept. of Agriculture* (1920); H. J. Quayle "Fumigation with liquid Hydrocyanic Acid." *Bulletin 308, University of California Experiment Station* (1919).

The fumigation of plants and trees by hydrocyanic acid not only eliminates injurious insects pests, but also has the remarkable

property of stimulating plant growth. This effect is exhibited not alone by the plant, but is also shown in the case of seed fumigation where a marked stimulation of germination results. For a discussion of the mechanism of this action and a bibliography see Moore and Willaman (*J. Agr. Res.*, 1917, **11**, 319). Clayton (*Bot. Gaz.*, 1919, **67**, 483) has studied this stimulating action in the case of the tomato, a plant which is particularly sensitive towards hydrocyanic acid.

In California, where the citrus groves are localised in a limited area, liquid hydrocyanic acid is transported by motor truck in 20-gauge tinned iron drums holding about eighty pounds. For shipment by rail the Bureau of Explosives of the Interstate Commerce Commission has authorised the use of seamless steel cylinders provided with a special valve and protection cap. Commercial cylinders used in the United States have a capacity of 30 or 75 pounds. Cylinders shipped under I.C.C. regulations bear a green label "Hydrocyanic Acid, Poisonous." In Germany, hydrocyanic acid absorbed in diatomaceous earth has found a considerable use in domestic and industrial fumigation.

Hydrocyanic Acid in Medicine.—Hydrocyanic acid is rarely used in medicine. Diluted hydrocyanic acid, according to the Pharmacopoeia of the United States (9th Revision 1916), is an aqueous solution containing not more than 2.1% HCN nor less than 1.9%, and not more than 0.1% HCl. U.S.P. No. 9 does not give a method for making diluted hydrocyanic acid, but U.S.P. No. 8 gives the following procedure: Mix 15.54 c.c. of diluted hydrochloric acid (sp. gr. 1.05) with 44.1 c.c. of distilled water; add 6 grams silver cyanide, and shake the whole together in a glass stoppered bottle. When the precipitate has subsided pour off the clear liquid.

According to the British Pharmaceutical Codex (1923) diluted hydrocyanic acid is an aqueous solution containing 2% by weight of hydrocyanic acid. It is obtained by interaction between diluted sulphuric acid and potassium ferrocyanide and subsequent distillation. Formerly the medicinal acid was of different strengths, as ordered by different pharmaceutical authorities, but the United States and the British Pharmacopoeias are now in accord. Scheele's or Stronger Hydrocyanic acid (*Acidum Hydrocyanicum Fortius*) is an aqueous solution containing 4% real hydrocyanic acid. It is used for poisoning animals and seldom in medicine.

Of the vegetable preparations containing hydrocyanic acid, the following are discussed in the United States Pharmacopoeia.

Oil of bitter almonds (Oleum Amygdalae Amarae), a volatile oil obtained by maceration and distillation from the ripe kernels of *Prunus Amygdalus amara de Condolle* and other kernels containing amygdalin. It yields not less than 85% of benzaldehyde, and not less than 2% or more than 4% HCN. It is a clear colourless or yellow, strongly refracting liquid, with the characteristic odour and taste of benzaldehyde; slightly soluble in water, soluble in all proportions in alcohol and ether. It dissolves, forming a clear solution, in 2 volumes of 70% alcohol; the specific gravity is 1.038 to 1.060 at 25°; the refractive index is 1.5428 to 1.5439 at 20°. When first prepared it is neutral to litmus, but becomes acid through formation of benzoic acid. The bitter almond oil of the British Codex (1923) contains not less than 2% or more than 4% HCN. The specific gravity should be 1.045 to 1.070, its boiling point 179°, and its refractive index 1.541 to 1.547.

Bitter almond water of both the American and British Pharmacopoeias is made by dissolving 1 c.c. of bitter almond oil by agitation in 999 c.c. of distilled water. The preparation contains a mere trace of hydrocyanic acid.

Cherry laurel water is not official in the United States. According to the British Codex it is prepared by boiling 80 gm. of cherry laurel leaves in a retort with 250 gm. of water and continuing until 100 c.c. are distilled. The strength of the finished product is adjusted by adding HCN or diluting with distilled water, so that the final hydrocyanic acid content is 0.1%.

Physiological Action of Hydrocyanic Acid.—Hydrocyanic acid is an active poison to all breathing organisms. Pure hydrocyanic acid has but little odour and is therefore not readily detected by the sense of smell at low concentrations. Long exposure to low concentration of the gas causes reddening of the eyes, followed by headache. At higher concentrations dizziness results, followed by darkening of vision and almost complete cessation of respiration. The action (Ewan, *Thorpe's Dictionary of Applied Chemistry*, 1921) seems to be a direct effect on the nerve centres and partly an inhibiting action on the enzyme of the red blood corpuscles. The poison is absorbed by the membranes of lungs or stomach, or even by the unbroken skin of the hands and arms.

Grubbs (*Public Health Reports*, 1917, 32, 565) states that men breathing quietly may sometimes breathe concentrations of 0.5 ounces of hydrocyanic acid per thousand cubic feet (400 parts HCN per million parts air) for two minutes without effect. Rapid breathing resulting from physical exercise causes the effect of the hydrocyanic acid to manifest itself. Grubbs considers the limit of safety for spaces in which work must be done to be 0.024% HCN (240 parts per million).

Lehmann (Kobert's *Kompendium der Praktischen Toxikologie*, 1912) gives the following data:—

Parts per million	Effect
20-40	Slight symptoms after several hours.
50-60	Serious disturbances in $\frac{1}{2}$ to 1 hr.
120-150	Dangerous in $\frac{1}{2}$ to 1 hour.
300	Quickly kills men and animals.

Katz and Longfellow of the U. S. Bureau of Mines (*J. Ind. Hygiene* 1923, 5, 97) recommend that permanent occupancy of ships or buildings which have undergone fumigation be forbidden until the residual hydrocyanic acid has fallen below 25 ppm.

Experience with men engaged in fumigation shows that a certain amount of tolerance to the gas is acquired. Chronic poisoning from continued exposure to very low concentration has been reported (Rosenbloom, *J. Lab. Clin. Med.*, 1923, 8, 258), but the experience of commercial producers would indicate that such cases are exceedingly rare. Reed (*J. Lab. Clin. Med.*, 1920, 5, 512) found no chronic symptoms in the case of dogs and no increased susceptibility as a result of repeated exposure to the gas. Some degree of tolerance seemed to result.

Since birds and small animals are frequently used as indicators of the respirability of the air of fumigated spaces, it should be noted that they exhibit a wide range of susceptibility. Birds are particularly sensitive to low concentrations. Mice and rats are also sensitive, and their use for test purposes is recommended by Grubbs (*loc. cit.*). Rats are slightly less susceptible than mice. Cats may also be used. According to Flury and Heuener (*Biochem. Z.*, 1919, 95, 249) cats may breathe gas of a concentration of 0.1 mg. per litre for some time without fatal results, though sometimes severe symptoms occur in 5 to 15 minutes. The lethal concentration for cats is

stated to be 0.35 mg. per litre (270 parts per million). 0.04 mg. HCN per litre (30 parts per million) had no injurious effects.

The analyst is occasionally required to report upon the suitability of fumigated foods for human use. No definite standards have been set up on which acceptance or rejection can be based. The United States Public Health Service (*Public Health Reports*, 1920, 35, 1597) has investigated the possibility of dangerous retention of hydrocyanic acid by fumigated food stuffs, the method consisting of exposing food to the action of hydrocyanic acid and then feeding animals with it. The conclusion drawn in this report is that the possibility of poisoning occurring from food materials exposed to hydrocyanic acid is extremely remote. Griffin and others (*U. S. Dept. Agric. Bull.* 1923, 1149), have determined the amounts of HCN absorbed and retained by fumigated foodstuffs, but they draw no conclusion as to the safety of such foods for consumption.

The lethal dose of cyanide through the mouth is variously stated. Potassium cyanide has caused death in doses of 13, 20 and 26 cgrms., but cases of recovery from as much as 2 and even 3 grm. are reported in the medical literature.

Inhalation of ammonia has been found to be one of the most effective remedies for the restoration of persons overcome by inhalation of hydrocyanic acid. For stomach poisoning with cyanides freshly precipitated ferrous carbonate is recommended. The emergency antidote consists of 50 c.c. portions of solutions containing 150 grm. of copperas ($\text{FeSO}_4, 7\text{H}_2\text{O}$) and 57 grm. of sodium carbonate per litre, preserved separately in stoppered bottles. The two reagents are to be mixed at the time of administration. Ewan (*loc. cit.*) states that he has seen an immediate cure effected in the case of dogs poisoned with cyanide, in which the poisoning had progressed to paralysis of the animal's hind quarters.

Qualitative and Quantitative Analysis of Hydrocyanic Acid and the Simple Cyanides

Detection of Hydrocyanic Acid.—Hydrocyanic acid and the soluble simple cyanides may be detected by a number of characteristic reactions, many of which are of great delicacy. Before applying any qualitative test it is preferable to obtain the hydrocyanic acid in a comparatively pure solution. This is best accomplished by acidification of the unknown with tartaric acid and distillation, the

qualitative tests being applied to the distillate. In presence of sulphides a little lead carbonate should be added to the mixture before distillation. Some of the most familiar tests are as follows:

(a) *Silver Nitrate Test*.—In 10 c.c. of solution 0.004 mg. of CN can be detected by addition of silver nitrate in presence of a drop or two of nitric acid. The precipitate of silver cyanide does not darken in sunlight. The test is made still more delicate by use of Chelle's colloidal silver iodide reagent (*Bull. Soc. Pharm. Bordeaux*, 1910, 70). To each 1 c.c. of the hydrocyanic acid solution is added 0.2 c.c. of colloidal silver iodide reagent. The reagent is made up by adding to 10 c.c. of 0.001*N* silver nitrate solution, 2 c.c. of strong ammonia and 5 drops of 10% potassium iodide solution and making up to 100 c.c. If the liquid remains cloudy, less than 0.001 mg. of HCN is present. With 0.005 mg. HCN more than 1 c.c. is necessary to obtain permanent cloudiness.

(b) *Prussian Blue Test*.—To 2 c.c. of the solution under examination add 2 drops of a 10% solution of ferrous sulphate and 2 or 3 drops of 10% caustic soda solution. Warm the mixture at about 50° for five minutes, add one drop of ferric chloride solution, and then acidify with a few drops of hydrochloric acid. A green coloration or a blue precipitate results, depending upon the amount of hydrocyanic acid present. According to Anderson (*Z. anal. Chem.*, 1916, 55, 459) 0.04 mg. of CN in 10 c.c. of solution will give the reaction.

(c) *Thiocyanate Test*.—Add to the cyanide solution a solution of an alkaline sulphide, drop by drop, until the yellow coloration persists. Then add a few drops of 10% caustic soda and evaporate to dryness. Take up with water and add 2 drops of dilute hydrochloric acid followed by 2 drops of a ferric salt solution. This test will detect the presence of 0.004 mg. of CN per 10 c.c. of solution tested (Anderson, *loc. cit.*). The test becomes even more delicate if the liquid is agitated with ether; the ferric thiocyanate dissolves in the ether and colours it red.

(d) *Colorimetric Tests*.—Three additional colour tests for hydrocyanic acid are described in a subsequent section on the Detection and Estimation of Hydrocyanic Acid in Air. These tests are the Picric Acid Test of Guignard, the Guaiacum and Copper Sulphate test of Schönbein and Pagenstecher, and the Phenolphthalin Test of Thiery.

Estimation of Hydrocyanic Acid.—*General.*—The specific gravity-concentration table of Hulbirt and Gray or of Walker and Eldred shown (on pages 483 and 486) is used for the estimation of liquid hydrocyanic acid by the specific gravity method. The gravimetric and volumetric methods used for the determination of hydrocyanic acid and its simple salts are discussed under the analysis of the Simple Metallic Cyanides (pp. 503 to 506). Certain special determinations are discussed below.

Determination of the Hydrocyanic Acid Content of Material Containing Cyanogenetic Glucosides

(Kohn-Abrest., *Ann. Fals.*, 1920, 13, 482). The sample, weighing about 50 gm., is ground and mixed with ten volumes of water in a flask having a capacity four or five times as great as the volume of the mixture. It is allowed to stand for four hours at 37° – 40° , or for 24 hours at room temperature, cooled and acidified with 2 c.c. of concentrated hydrochloric acid for every 100 c.c. of liquid. The flask is then connected with a condenser the end of which dips into a few c.c. of water, and the distillation is carried on in a calcium chloride bath until about one-fourth of the mixture has distilled. The distillate is rendered slightly acid and solid sodium bicarbonate is added, after which the solution is titrated with a standard solution of iodine in potassium iodide. The residue from the distillation is treated with a volume of concentrated hydrochloric acid equal to $\frac{1}{10}$ of the original volume and the distillation is continued until $\frac{3}{5}$ of the original volume have been collected. The second distillate is titrated as above. This gives the hydrocyanic acid which has not been liberated by the maceration in cold water.

Assay of Oil of Bitter Almonds (U. S. Pharmacopoeia 10th Revision). Dissolve 0.75 gm. magnesium sulfate in 45 c.c. of distilled water, add 5 c.c. half normal sodium hydroxide and two drops of potassium chromate T. S., and titrate the solution with tenth normal silver nitrate to the production of a permanent reddish tint. Pour the mixture into a 100 c.c. flask containing 1 gm. of oil of bitter almonds. Mix well and titrate again with 0.1N AgNO_3 until a red tint, which does not disappear on shaking, is reproduced. Conduct the titration as rapidly as possible. 1 c.c. of 0.1N AgNO_3 equals 0.0027 gm. of HCN. One gm. of the oil corresponds to not less than 7.4 c.c., and to not more than 14.8 c.c. of 0.1N AgNO_3 .

Estimation of Hydrocyanic Acid in Air.—Owing to the extensive use of cyanide compounds in metallurgy and to the rapidly increasing application of hydrocyanic acid as a fumigant, a simple method for the estimation of residual hydrocyanic acid in the air of tanks and apparatus and in buildings and ships after fumigation has been urgently needed. Although the literature contains an abundance of references to colorimetric tests for hydrocyanic acid, these tests are usually wholly qualitative and are not sufficiently specific to serve as positive tests for the respirability of air by human beings. Recent studies by Katz and Longfellow of the Bureau of Mines, U. S. Department of the Interior (*loc. cit.*) have placed these tests on a sound quantitative basis, and in the following paragraphs we quote extensively from their findings (*J. Ind. Hygiene*, 1923, 5, 97).

Three tests were selected as best adapted to colorimetric measurements. (1) Picric acid with sodium carbonate (Guignard's Test); strips of filter paper are impregnated with sodium picrate solution and dried. In presence of hydrocyanic acid a colour change from yellow through orange to copper occurs. (2) Guaiacum and copper sulfate (Schönbein and Pagenstecher's Test). Test papers impregnated with the reaction mixture turn from colourless to deep blue in presence of hydrocyanic acid. (3) Phenolphthalin and copper sulfate and sodium hydroxide (Thiery's Test). The change here is from colourless to deep pink. (Note that phenolphthalin is to be distinguished from phenolphthalein, the familiar indicator in acidimetry.) A very complete bibliography of the literature of these three tests is given in the original article by Katz and Longfellow.

The work of Katz and Longfellow related particularly to the detection and estimation of hydrocyanic acid over a range of 25 to 1,000 volumes of hydrocyanic acid per one million volumes of the mixture with air. By a combination of the three colorimetric tests they developed a formal procedure for testing which would be applicable under all possible contingencies. This procedure and the apparatus required for its application are as follows:

The complete apparatus consists of the following parts.

(1) Standard colour chart made by mounting twelve small strips of coloured paper described in the table on page 497 on a card. Under

each shade the concentration of hydrocyanic acid and the time of exposure to produce the shade is indicated. (2) Bottle containing strips of filter paper, $\frac{3}{4}$ inch wide and 2 inches long. (3) Bottle containing dry filter paper strips previously wet with the solution of copper sulphate, as described below. (4) Bottle containing sodium picrate papers, prepared not more than one week previous to use, by dipping them into the sodium picrate solution described below and then drying them in room air. (5) Bottle of copper sulphate solution; 50 mg. in 100 c.c. of water. (6) Bottle of tincture of guaiacum; 4 grm. of the guaiacum in 100 c.c. of alcohol. (7) Bottle of alkaline phenolphthalin solution, made as follows: Dissolve 20 grm. of sodium hydroxide in 100 c.c. of water, stir in 0.5 grm. of phenolphthalein dissolved in 30 c.c. of alcohol, put into a 12 inch evaporating dish, add 25 grams of aluminum, about 30 to 60 mesh powder; heat and add water as necessary to continue the reaction. After 20 minutes to one hour, when the solution is colorless, filter with suction; dilute to 250 c.c. and preserve. (8) Bottle of sodium picrate solution, 1 grm. of picric acid and 10 grm. of sodium carbonate dissolved in 100 c.c. of water. (9) Three wooden clips to hold papers during tests. A wire support mounted in the lid of the box used to carry the apparatus is provided to support the clips.

The directions for simultaneous testing with three test papers are designed for use by an operator wearing a gas mask with soda-lime cannister. Similar procedure may be followed when the observer is outside the space under examination; in this case the test papers are attached to a stick which is pushed into the gas space.

(1) Set up the wire support for the test papers, lay the colour chart beside the support, lay a watch beside the chart, and loosen the stoppers of the bottles. (2) Observe the second hand of the watch and at zero second remove a picrate paper, re-stopper the bottle, and suspend the paper from the wire support with a clip. (3) While observing the picrate paper, time and colour chart, put a copper sulphate paper in a clip, and at the zero second dip into phenolphthalin solution, re-stopper immediately, shake off excess solution and suspend the paper. (4) Continue to observe the tests, place another copper sulphate paper in a clip at the next zero second, dip into the guaiacum tincture, and suspend the papers; observe the guaiacum during its first minute and all papers at each minute thereafter until the picrate paper has had five minutes exposure. (5) Make

final decision of concentration of hydrocyanic acid. Concentrations of 100 parts per million are safe for one hour; 500 parts per million are dangerous. Unprotected persons are permitted to enter a space with a hydrocyanic acid concentration not exceeding 100 parts per million.

CHART SHOWING COLOURS DEVELOPED BY EXPOSURE OF TEST PAPERS TO HYDROCYANIC ACID IN AIR

Test papers	Concentration—HCN in p. p. m.	Time of exposure in minutes	Color Mulliken's standard symbol for color shade*	Description of commercial paper to match
Sodium picrate (dry)	0	Yellow normal tone	Canary Uncle Sam Bond. Alling & Cory Co., Pittsburgh, Pa.
	25 100	4 1	Orange yellow normal tone Orange yellow normal tone	Golden Rod Hammermill Bond. Hammermill Paper Co., Erie, Pa.
	100 500 1,000	5 2 1	Orange yellow shade 2 Orange yellow shade 2 Orange yellow shade 2	Twentieth Century Rope Bristol. Alling & Cory Co., Pittsburgh, Pa.
	500 1,000	5 3	Yellow orange shade 2 Yellow orange shade 2	Copper Ant Bay Bath cover. Strathmore Paper Co., Mit-tineaque, Mass.
	0	White
Guaiacum-copper (wet)	25 50	1 1/4	Green-blue tint 2 Green-blue tint 2	Blue Liberty Cover Knowlton Bros., Watertown, N. Y.
	25 100 500	3 1 1/4	Green-blue tint 1 Green-blue tint 1 Green-blue tint 1	Opalina Blue Manuscript Cover. Alling & Cory Co., Pittsburgh, Pa.
	100 500 1,000	3 1/2 1/4	Green-blue normal tone Green-blue normal tone Green-blue normal tone	Blue Highland Blotting. Alling & Cory Co., Pittsburgh, Pa.
	0	White
	25 100	4 1	Violet red tint 4 Violet red tint 4	Pink Empire Bond, Carew Mfg. Co., South Hadley, Mass.
Phenolphthalin copper (wet)	100 500	4 1	Violet red tint 2 Violet red tint 2	Pink Arena Bond, Alling & Cory, Pittsburgh, Pa.
	500 1,000	2 1	(Between red violet and violet red tint 1)	Papier Nouvelle No. 2013. Coyle & Gilmore, 5th Ave., N. Y.

* S. P. Mulliken, *Identification of Pure Organic Compounds* (1908), John Wiley and Sons, New York.

It has been found that none of the test papers keeps well, because the colours develop slowly. Guaiacum paper prepared, dried, and kept in a closed bottle exposed to light changes to a shade of blue

comparable with the first shade of the chart in one or two days. Phenolphthalin paper keeps a little better. The copper sulphate impregnated paper used for the guaiacum and phenolphthalin tests may be made, dried and kept indefinitely. Picrate papers were found more reactive when freshly made. They may be kept for not more than a week after preparation for good results; eventually they darken, probably due to reduction caused by the cellulose. Guaiacum paper is sensitive only when wet; the others react wet or dry, but the picrate is best dry.

The action of interfering gases was also studied. Their behaviour toward the various papers was as follows:

Picrate paper was changed by hydrogen sulphide only; the change was inhibited by sulphur dioxide.

Guaiacum paper was changed by chlorine, nitrogen peroxide, tobacco smoke, ammonia and formaldehyde; the change was inhibited by sulphur dioxide.

Phenolphthalin paper was changed by ammonia and nitrogen peroxide; the change was slightly inhibited by sulphur dioxide.

Thus, although several other gases act upon one or two papers in the same manner as hydrocyanic acid, there is no possibility of faulty indications by all three of the indicators except when sulphur dioxide is present. Since sulphur dioxide is readily detected by its irritant action upon the eyes and nose at fifty parts per million, and by its odour even at concentrations as low as five parts per million, there is little possibility of serious error resulting from its presence.

The present writer had much experience in the application of the procedure of Katz and Longfellow. In connection with the determination of hydrocyanic acid in fumigating chambers a more precise method was required, and it was found that, of the three tests, the sodium picrate test was capable of most exact standardisation. It will be noted from Katz and Longfellow's table that the depth of colour produced by hydrocyanic acid is a function of the "exposure units" (parts per million times minutes of exposure) to which the picrate paper had been subjected. A series of colour standards was prepared, the individual colours being so chosen that the "exposure units" for each succeeding colour on the scale increased geometrically with a ratio of two. These standards may be referred to the Mulliken color chart, but for very accurate work the writer employs a special set of standards prepared by use of water colours.

The agreement between the picrate and hydrocyanic acid colours and the Mulliken chart is very good for the first and last third of the scale, but is not so satisfactory for the middle third.

CHART SHOWING COLOURS DEVELOPED BY EXPOSURE OF SODIUM PICRATE TEST PAPERS TO HYDROCYANIC ACID IN AIR

"Exposure Units" (ppm HCN \times minutes exposure)	Colour. Mulliken's standard symbol
0 75 150	Yellow. Normal tone. Orange-yellow, normal tone. Orange-yellow shade 1.
300 600 1,200	Yellow-orange shade 1. Yellow-orange broken tone. Yellow-orange shade 2.
2,400 4,800 9,600	Orange broken tone. Orange-red broken tone. Red broken tone.

Certain special precautions have been found necessary in the preparation and use of the picrate test papers. The picrate solution used by the writer is prepared by dissolving 5 gm. of picric acid and 50 gm. of sodium carbonate in one litre of water. The test papers are prepared by dipping long strips of Whatman's No. 1 filter paper into the picrate solution and then pressing between pads of blotting paper. The moist strips are then hung up to dry and are finally cut into strips, 1 cm. \times 8 cm., which are kept in stoppered bottles. The drying of the strips should not be done by artificial heat. For best results the papers should have a soft feel, and it is preferable to err on the side of too short rather than too long drying. Papers should be discarded if more than a week old.

The work of Katz and Longfellow was done at 50% relative atmospheric humidity, and they do not state what effect is caused by variation from the standard humidity. 50% relative humidity was also employed in the preparation of the colour standards used by the writer. In a study of the effect of variation of atmospheric humidity it was found that substantially correct results are secured when the papers are used in the air of rooms in which the humidity is from 40 to 75% of saturation. At higher humidities the test papers develop colours somewhat deeper than the standards, thus

yielding results that are slightly high. In the case of humidities much below 40% the papers may be entirely misleading; in very dry air little or no colour develops. This limitation of the sodium picrate test for hydrocyanic acid must always be kept in mind.

Estimation of Hydrocyanic Acid in the Body.—The detection of hydrocyanic acid in the body is rendered difficult by the great facility with which the acid decomposes. Calvi and Malacarne (*Giorn. farm. chim.*, (1906), **56**, 5) found that they could detect hydrocyanic acid in the human intestines 22 days after death; after 30 days only traces could be found. The decomposition of the acid is retarded in the presence of 95% alcohol and it is therefore recommended that parts intended for analysis be preserved with alcohol. Autenrieth (*Ber. Pharm. Ges.*, 1910, **20**, 432) was able to identify hydrocyanic acid in an exhumed corpse 43 days after burial. In six quantitative experiments on the stability of hydrocyanic acid in putrefaction, in which mixtures of animal stomach, intestines and blood with bitter almond water or potassium cyanide were used, the hydrocyanic acid recovered after sixty days varied from 41.4 to 63.3%. The method used was distillation of the mixture after acidifying with tartaric acid, and titration of the distillate, after addition of 6–8 drops of caustic potash, with 0.05*N* silver nitrate.

On opening the stomach and intestines the odour of hydrocyanic acid is often perceptible. These viscera are often quite natural in appearance, but are sometimes more or less inflamed and congested. The lungs, liver, spleen and kidneys are always found gorged with blood. The venous system is invariably gorged with blood, the arteries being empty. The blood has undergone change; it may be black and oily, or a cochineal-red colour. It often smells of the poison, which frequently may be distilled from it.

To detect hydrocyanic acid in the contents of the stomach the analyst should proceed as follows:—

Note the reaction of the liquid portion. If not distinctly alkaline to litmus, the poison (if present) was probably administered as free hydrocyanic acid and not as an alkali cyanide.

Stir the stomach and its contents with cold water and introduce the thick liquid into a flask adapted to a Liebig condenser, allowing the end of the condenser to be immersed in a small quantity of

water. Apply a moderate heat to the flask (best by an external bath of salt water) and distil over about half the liquid. To the distillate apply the silver nitrate, Prussian blue, and thiocyanate tests as described on page 493. It is preferable to avoid any addition of acid to the liquid to be examined, as the saliva contains traces of thiocyanates, which might possibly yield traces of hydrocyanic acid on distillation with mineral acid. If the distillate has given negative results when tested for hydrocyanic acid, continue the distillation after rendering the contents of the flask distinctly acid with tartaric acid. If hydrocyanic acid is now found in the distillate, the poison must have been present as a readily decomposable cyanide. The guaiacum and copper reaction (page 495) is recommended as a preliminary test. If the distillate gives no indication of hydrocyanic acid by this test, it is considered useless to proceed further; if an affirmative result is obtained, other tests should be employed. If only a small quantity of hydrocyanic acid is present in the distillate, it is most conveniently detected by precipitating with silver nitrate and applying the ferric thiocyanate test to the precipitate (page 493). The blood-red thiocyanate colour is distinguished from that due to an acetate or a formate by being unaffected by dilute hydrochloric acid, and from that produced by a meconate by being readily destroyed on addition of mercuric chloride. Before finally concluding that all metallic cyanides are absent it is desirable to repeat the distillation after adding a considerable excess of moderately dilute sulphuric and hydrochloric acid. Ferrocyanides, ferricyanides and mercuric cyanide will in this case be decomposed. Hence the absence of ready-formed ferrocyanides or ferricyanides should be previously ascertained by testing portions of the acidified contents of the stomach with solutions of ferric and ferrous salts.

Owing to the instability of ferrocyanides in acid solutions, Autenrieth (*Arch. Pharm.*, 1893, 231, 99) considers that the only certain way to detect hydrocyanic acid and simple cyanides in their presence is by Jaquemin's process. He distils the material with a considerable amount of sodium hydrogen carbonate and examines the distillate for hydrocyanic acid. If the presence of mercuric cyanide is suspected, hydrogen sulphide water must also be added, as the sodium hydrogen carbonate does not itself decompose mercuric cyanide. Mercuric cyanide may also be decomposed by adding a

few bright strips of zinc to the liquid before commencing the distillation. Ferricyanides, thiocyanates, sulphates and ammonium salts do not interfere with the process.

Mercuric cyanide may be decomposed by distillation with hydrochloric acid. After testing for other simple cyanides, mercuric cyanide can be estimated by acidifying the mixture with tartaric acid, after distillation with sodium hydrogen carbonate, adding ammonium chloride in excess and again distilling. A double chloride of mercury and ammonium is said to be formed, and hydrocyanic acid distils with the steam. To test for cyanides in presence of ferrocyanides Lopes heats the substance to 100° with milk of lime in order to decompose ammonium salts, which, if present, may react with ferrocyanide to form volatile ammonium cyanide. When all the ammonia has been driven off the solution is filtered and distilled with an excess of sodium hydrogen carbonate, as recommended by Autenrieth.

One of the main causes for the disappearance of hydrocyanic acid in the organs is the formation of thiocyanates from the hydrogen sulphide formed by putrefaction. Thiocyanate also eventually disappears. Chelle (*Bull. Soc. Pharm. Bordeaux*, 1919, **57**, 228) has made a study of the disappearance of hydrocyanic acid under these conditions, and finds that it is necessary to determine both unaltered hydrocyanic acid and also thiocyanate. His method as applied to blood is as follows: To 40 grms. of blood add sufficient 5% solution of picric acid to make 100 c.c. and filter. Place 50 c.c. of the filtrate in a flask, add 5 c.c. of 50% sulphuric acid and aerate for two hours, scrubbing the air with 0.1N KOH solution to absorb the liberated hydrocyanic acid. When this is completed add an excess of a 20% solution of potassium dichromate and aerate again. The hydrocyanic acid liberated in the second operation is derived from the thiocyanates. The hydrocyanic acid liberated in each treatment is determined by titration with a 0.0001N ammoniacal silver iodide solution according to the method described on page 493.

For the examination of organs and tissues Chelle places a 40 gm. sample of the finely chopped material in a flask, adds 75 c.c. of distilled water and 5 c.c. of phosphoric acid and distils. Free hydrocyanic acid is determined in the distillate as described above. For the thiocyanate determination the residue in the flask is made up to 100 c.c. with aqueous picric acid, the liquid filtered, and hydro-

cyanic acid liberated by addition of potassium dichromate and sulphuric acid.

Since thiocyanate is normally present in the organism, its identification is not evidence of hydrocyanic acid poisoning. Chelle (*ibid.*, 1920, 58, 20) reports the following thiocyanate contents of normal individuals.

Ten samples of saliva.....	17.3 to 217	mg. HCNS per liter.
Ten samples of urine.....	0.0 to 6.6	mg. HCNS per liter.
1 sample gastric juice.....	7.1	mg. HCNS per liter.

Negative results were obtained with normal specimens of pancreatic juice, milk and blood. The blood of a person fatally poisoned with carbon monoxide contained 12.4 mg. HCNS per litre.

Analysis of the Simple Cyanides.—*Gravimetric Methods.*—The gravimetric estimation of cyanogen in cyanides is carried out as follows:—Dissolve a weighed quantity of the cyanide in water, and slightly acidify the solution with nitric acid. An excess of nitric acid is to be avoided on account of the solubility of silver cyanide in this reagent. To this solution add silver nitrate (5% solution), drop by drop and with constant stirring, until precipitation is complete. Stir the mixture vigorously to coagulate the precipitate but do not heat, allow the precipitate to settle and then filter on a tared Gooch crucible. Wash the precipitate, dry at 100°, and weigh as AgCN. The weight of the silver cyanide may be checked by igniting it at a red heat for fifteen to twenty minutes. Metallic silver is liberated and may be weighed as such.

This method is, of course, applicable only to mixtures which contain no acid radical, except cyanide, which is precipitated by silver. If sulphides are present, they must be removed by a preliminary shaking with freshly precipitated lead carbonate.

In the presence of chlorides, bromides and iodides the precipitated silver cyanide must be separated from the silver halides. This can be conveniently accomplished by boiling the precipitate with a solution of mercuric acetate, in which the silver cyanide will dissolve, forming silver acetate and mercuric cyanide. The solution is then filtered, thus separating the cyanide from the silver halides. The silver in the filtrate is estimated by precipitating it as silver chloride and then reducing the silver chloride in a current of hydrogen gas to silver. It is important to reduce the silver chloride instead of weighing the silver chloride direct, because, when the silver is

precipitated, it is liable to bring down some mercury with it, which, being enclosed in the precipitate, does not volatilise when the silver chloride is heated.

Liebig's Volumetric Method.—The Liebig method for the estimation of simple cyanides is the procedure usually applied in commerce on account of its simplicity and accuracy. Liebig's method is based on the fact that silver cyanide forms a soluble double salt with an aqueous solution of an alkali or an alkaline earth cyanide.—



On the addition of an excess of silver nitrate the double salt is decomposed and silver cyanide is precipitated; a drop of the silver solution in excess causes a permanent turbidity of the solution. This method only indicates the metallic cyanide present, free hydrocyanic acid alone giving an immediate precipitate with silver nitrate. Hence, if free hydrocyanic acid is present, the solution must first be treated with a full equivalent of alkali hydroxide, but any great excess should be avoided, as the results may be rendered high. However, the solution at the termination of the titration should be distinctly alkaline to litmus, otherwise more sodium hydroxide must be added (which will cause the disappearance of the turbidity) and then the titration continued till a permanent turbidity results.

If alkali sulphides are present, they must be separated before the titration starts, as the slightest amount of sulphide is detected in titrating by the formation of a yellow to brown colour in the solution. The sulphides are precipitated from the alkali cyanide solution by the addition of freshly precipitated lead carbonate, the insoluble sulphides being filtered off and washed.

The sharpness of the titration is increased and the interference of excess alkali is eliminated by the addition of a few drops of potassium iodide solution before titrating. In this case the end-point turbidity is of a yellowish hue instead of bluish-white. The precipitate of silver iodide which indicates the completion of the titration is insoluble in caustic alkalis and in ammonia; hence the titration may be made with accuracy in their presence. The use of iodide indicator in the Liebig titration is almost universal.

In a liquid containing alkali cyanide as well as free hydrocyanic acid, the amount of each may be ascertained by titrating first without addition of alkali hydroxide, and then continuing the process after

adding it. The volume of the silver solution first used represents the metallic cyanide; the second quantity, the free hydrocyanic acid.

The usual procedure for the estimation of the cyanide content of an alkali cyanide is as follows: Dissolve 2 grm. of the sample in approximately 150 c.c. of water, add a little freshly precipitated lead carbonate and 10 c.c. of 10% caustic soda solution. Shake thoroughly and test for complete precipitation of sulphides by addition of a little more lead carbonate. Filter into a 250 c.c. volumetric flask, wash and make up to volume. After thorough mixing pipette out 50 c.c. of the solution, dilute to about 300 c.c. and add five drops of 10% KI solution. Place the beaker on a black surface and run in $N/10$ silver nitrate solution from a burette, with continual agitation, until a permanent opalescence appears.

In commercial practice it is customary to simplify this procedure by neglecting the volume of the solid lead salts. In this case the mixture is made up to volume without filtering, poured on to a dry filter, and a 50 c.c. aliquot portion of the clear solution titrated.

The standard silver nitrate solution is prepared by dissolving 16.99 grm. of pure dry silver nitrate in water and making up to one litre. One c.c. of this solution is equivalent to 0.0098 grm. of sodium cyanide by the Liebig titration, or to 0.00355 grm. of chlorine by the Volhard or Mohr titration. Sufficient accuracy for ordinary work is attained by weighing out the dry silver nitrate; where greater precision is desired the silver solution may be standardised either gravimetrically or volumetrically against pure sodium chloride.

Nickel Titration.—The nickel titration of Lundell and Bridgman (*J. Ind. Eng. Chem.* 1914, 6, 554) is based upon the titration of an ammoniacal hydrocyanic acid solution with nickel ammonium sulfate in the presence of dimethylglyoxime. The characteristic red coloration of the nickel dimethylglyoxime appears only after all the cyanide has combined as the nickel double salt. The reaction is—



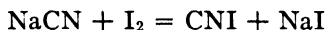
No permanent red precipitate of nickel dimethylglyoxime is formed until all the cyanide has been used up according to this equation.

The reagents consist of a solution of 15.3 grm. of nickel ammonium sulphate in 1,000 c.c. water, to which are added 2 c.c. of concentrated

sulphuric acid. 100 c.c. of this solution are equivalent to 1 grm. of KCN. The indicator is a solution of 8.9 grm. of dimethylglyoxime in 1,000 c.c. of 95% alcohol.

To carry out the titration, approximately 0.5 grm. of the sample, dissolved in 100 c.c. of water, is treated with 1 c.c. of ammonium hydroxide and 0.5 c.c. of the dimethylglyoxime solution, and the mixture is titrated with the nickel solution until a permanent red precipitate is produced.

Iodine Titration. (*Method of Fordos and Gelis*).—Use of this titration for the determination of hydrocyanic acid in cyanogenetic glucosides has already been described (page 494). The method is based on Serrula's and Wöhler's reaction—

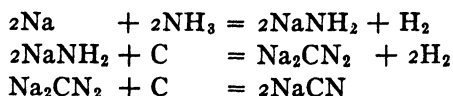


Sulphides must be absent and free alkalis or ammonia must be neutralised by addition of an excess of carbonated water or of a bicarbonate.

The Simple Metallic Cyanides

The simple cyanides are all decomposed by boiling with dilute hydrochloric acid, hydrocyanic acid being liberated quantitatively. All of them exhibit the characteristic property of entering into double-salt combination with other salts and with each other. In the following section we discuss the properties of those simple metallic cyanides which have industrial importance, paying particular attention to the aspects of each which are of special interest to the analytical chemist.

Sodium Cyanide.—The sodium cyanide of commerce is almost exclusively produced by the Castner process, in which metallic sodium and gaseous ammonia are caused to react in presence of carbon. A series of reactions occurs in which sodium amide and sodium cyanamide are intermediate products, the final product consisting of a fused sodium cyanide of high purity. The reactions occurring are as follows:



The fused cyanide is finally cast into moulds and appears in commerce as slabs or blocks, or as cylindrical or egg-shaped lumps.

Being made from pure raw materials, the Castner process cyanide is usually of white, porcelain-like appearance and is very pure. The usual commercial guarantee is for a sodium cyanide content of 96 to 98%, the remainder consisting of small amounts of cyanate, carbonate and, occasionally, cyanamide. Material is occasionally encountered which is decidedly off-colour, being black or gray from finely dispersed carbon.

A smaller amount of sodium cyanide produced by wet processes also appears on the market; it is produced by absorption of hydrocyanic acid in caustic soda solution and evaporation to dryness of the resulting solution of sodium cyanide. The powdered sodium cyanide so obtained is usually briquetted into blocks weighing about one pound each. The wet-process cyanides are inferior in appearance to the fused product of the Castner process and are also of lower cyanide content, (from 90 to 92% NaCN). They contain from 2 to 3% each of sodium formate and sodium carbonate, together with smaller amounts of caustic soda, sodium chloride, sodium cyanate, and other sodium salts. The most important wet-process cyanide is the by-product cyanide produced by absorption of the hydrocyanic acid liberated during the destructive distillation of beet sugar refuse.

Sodium cyanide is exceedingly soluble in water. When crystallised from water at temperatures below 34.7° it crystallises with two molecules of water. The solubility (gram. of NaCN in 100 gram. of water) is: 35.4 at -20° ; 43.4 at 0° ; 58.3 at 20° , and 82 at 34.7° . There is a transition-point at 34.7° C. (Ewan), and above this temperature the salt crystallises in the cubical system and without water. The solubility of the anhydrous salt is substantially independent of temperature.

The melting point of pure sodium cyanide, according to a recent determination by Grandadam (*Compt. rend.*, 1925, **180**, 1598), is 567.3° . The vapour pressure of the liquid has been determined by Ingold (*J. Chem. Soc.*, 1923, **123**, 885), who finds it to be 0.76 mm. at 800° C., 12.4 mm. at $1,000^{\circ}$, and 89.8 mm. at $1,200^{\circ}$.

The principal applications of sodium cyanide are in the mining industry, in electro-plating, for case-hardening steel, for fumigation, and for the manufacture of dyes and other chemicals. Its importance in the mining industry is much less than in former years, it having been superseded in this industry by the cheaper calcium

cyanide. In this respect history has repeated itself; potassium cyanide, which was at first exclusively used in the metallurgical industries, gave place to the cheaper sodium cyanide, which, in turn, has yielded to the still cheaper calcium cyanide.

The fact that potassium cyanide was originally the important commercial alkali cyanide has given rise to a somewhat confusing trade usage. The sodium cyanide "96-98%" of the American market is quoted on the English market as "128-130%," the units in the latter case being calculated on a KCN basis. In the same way the 90% wet-process product is designated 120% in the KCN system. Quotations in English trade publications are made on the basis of 100% KCN; to convert such quotations to the NaCN basis they must be multiplied by the factor 1.33.

Potassium Cyanide.—Although originally the best known of the metallic cyanides, potassium cyanide is now of minor importance in the arts. Only in a few special instances, as for example, in certain electro-plating baths and in special chemical manufactures, has it maintained itself against the competition of the cheaper cyanides of sodium and of calcium.

The alkali cyanides, both of sodium and potassium, were originally produced by the fusion of the corresponding ferrocyanide, decomposition taking place according to the reaction—



To avoid the excessive loss of cyanogen which occurs with this method Liebig recommended the addition of alkali carbonate to the ferrocyanide before fusion. The reaction in this case is—



Finally, Erlenmeyer proposed fusion of the ferrocyanide with metallic sodium. Thus with sodium ferrocyanide the reaction is—



When potassium ferrocyanide is substituted for the sodium salt in this reaction the resulting product is a mixture containing 4 molecules of potassium cyanide to 2 molecules of sodium cyanide.

All the processes mentioned in the preceding paragraph are obsolete so far as sodium cyanide manufacture is concerned; they are still sometimes used for the production of the potassium salt. The

potassium cyanide of commerce may be a mixture of the cyanides of sodium and potassium as produced by the Erlenmeyer process, or it may be free from sodium cyanide and prepared either by fusion of potassium ferrocyanide or by wet processes followed by fusion. The more usual grade of potassium cyanide on the American market contains 90 to 92% KCN.

Pure potassium cyanide may be prepared by crystallisation from alcohol or water. The aqueous solubility curve differs materially from that of sodium cyanide. Solubility determinations show the following relation for the temperature interval 0° to 50° C:

$$\% \text{ KCN in saturated solution} = 39.7 + 0.11 t,$$

where t = temperature in °C. The anhydrous salt crystallises from water. It melts at 601° C. (Rassow *Z. anorg. Chem.*, 1920, **114**, 117) (or 634.5° C. according to Grandadam, *loc. cit.*) and solidifies, on cooling, to a white crystalline mass.

Examination of Commercial Alkali Cyanides.—Commercial alkali cyanides may contain, in addition to cyanide, the chlorides, carbonates, hydroxides, cyanates, sulphides and cyanamides of the alkali metals.

The *cyanogen content* is estimated by Liebig's volumetric method. To estimate the *alkali* about 0.5 gm. of the salt is dissolved in water, and the solution evaporated to dryness in a porcelain dish to which 5 c.c. of dilute hydrochloric acid (20%) have been added. The dry residue containing potassium and sodium chlorides is estimated in the usual way.

The *chlorides* are estimated volumetrically with $N/10$ silver nitrate. Enough $N/10$ solution is added to produce a permanent turbidity; then a further amount of silver nitrate is added exactly equal to the first addition. This provides the exact amount of silver nitrate necessary for complete precipitation of the cyanide as silver cyanide. A few drops of potassium chromate are now added to the solution, and the titration with $N/10$ silver nitrate is proceeded with until a pale red tint is observed which does not disappear after vigorous agitation. The last amount of $N/10$ silver nitrate used corresponds to the chloride present.

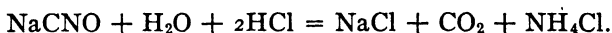
The *carbonates* are estimated by adding an excess of barium nitrate to the solution of the cyanide, placing the mixture in a well-closed flask, and allowing the barium carbonate to settle. The

barium carbonate is then filtered off and washed, precautions being taken to keep the air away as much as possible. The washed precipitate is dissolved in a known volume of standard hydrochloric acid, and the excess of acid back-titrated after boiling to remove carbon dioxide.

For the estimation of the *hydroxides* magnesium nitrate may be added to the filtrate from the barium carbonate precipitate, thus precipitating magnesium hydroxide, which can be filtered off, washed, and determined as MgO after ignition, or by titration. Clennell (*The Cyanide Handbook*) dissolves a weighed portion (about 1 grm.) in water, adds sufficient silver nitrate to give a permanent precipitate, then a few drops of an alcoholic solution of phenolphthalein, and titrates with standard acid. The result obtained is equivalent to the hydroxide *plus* half of the carbonate. Carbonate having been determined as previously described, the hydroxide content is obtained by difference.

The method for the determination of alkali *cyanate* is based on the solubility of silver cyanate in dilute nitric acid, whilst silver chloride and silver cyanide are insoluble. A known amount of alkali cyanide, (about 0.5 grm.) is dissolved in water, and enough barium and magnesium nitrates are added to precipitate, respectively, the carbonates and hydroxides present. After the precipitates have settled they are filtered off and washed. A neutral solution of silver nitrate is added to the filtrate until no more precipitate is formed. The precipitate, which consists of silver cyanide, silver chloride and silver cyanate, is allowed to settle; it is then filtered off and washed until no more silver can be detected in the wash water. The precipitate is washed into a beaker and stirred up with 200 c.c. of water to which 10 c.c. of dilute nitric acid (sp. gr. 1.2) have been added. It is then digested on the water-bath for one hour. The beaker must be covered with a watch-glass and the contents frequently agitated. The silver cyanate goes into solution, while the other silver salts remain undissolved. The solution is filtered from the residue, and the amount of silver in the filtrate is determined by Volhard's volumetric method. From the amount of silver in solution the alkali cyanate can be calculated. Unless the directions of this method are closely followed erroneous results will be obtained, owing to the appreciable solubility of silver cyanide in dilute nitric acid.

A more rapid and equally accurate method for the estimation of alkali cyanate has been described by O. Herting (*Z. angew. Chem.*, 1901, **14**, 585). 0.2 to 0.5 gram. of the salt is placed in a porcelain dish and dissolved in a few c.c. of water. Sufficient dilute hydrochloric or sulphuric acid is added to provide an excess, the dish is placed on a water-bath, and its contents evaporated to dryness. The cyanate is decomposed, with formation of an ammonium salt, thus:—



The residue is dissolved in water, and the ammonia is estimated by distillation with sodium hydroxide and absorption in standard acid.

For the determination of *sulphides* Ewan (*J. Soc. Chem. Ind.*, 1909, **28**, 10) titrates a solution containing 10 gram. of alkali cyanide with a standard lead nitrate solution until a drop of the solution, when brought in contact with a drop of lead nitrate solution on heavy filter paper used for drop reactions, no longer gives a brown stain.

The presence of *cyanamide* can be detected by adding an excess of strongly ammoniacal silver nitrate solution (10%) to a very dilute cyanide solution. If a trace of alkali cyanamide is present, a yellow precipitate of silver cyanamide is formed. If a too concentrated solution of cyanide is used, a beautiful white crystalline scale-like precipitate of ammonium silver cyanide is formed. The same reaction is employed for the estimation of alkali cyanamide. The yellow precipitate of silver cyanamide (silver chloride and silver cyanide being soluble in ammonia) is filtered off, and the cyanamide estimated as described on page 602.

Calcium Cyanide.—Until quite recently, calcium cyanide was known only in solution. Frank and Caro, attempting to produce calcium cyanide by passing nitrogen over calcium carbide, obtained calcium cyanamide but no cyanide. The modern calcium cyanamide industry is the outgrowth of these experiments. Erlwein and Frank subsequently patented a process for the conversion of calcium cyanamide into cyanide by fusion with a metallic chloride. Landis (*Can. Chem. J.*, 1920, **4**, 130) describes the arrangements employed at Niagara Falls, Ontario, where this process is conducted on a very large scale. Calcium cyanamide is fused with common salt in a

single-phase electric furnace, and the melted mass is tapped at intervals from the furnace and quickly chilled on a cooling wheel. The product thus obtained contains sodium, calcium, cyanogen and chlorine, and the question is frequently raised whether it is a mixture of sodium cyanide and calcium chloride, sodium chloride and calcium cyanide, or of all four of these compounds. Although no definite answer can be given to this question, the weight of the evidence is in favour of the calcium cyanide formula. The most convincing argument is the fact that the product now on the market contains very much less sodium than the equivalent of the cyanogen; it necessarily follows, therefore, that a considerable part, at least, of the cyanogen is combined with calcium. Experience with the application of this product in various arts has shown that its properties are in many respects different from those of the alkali cyanides.

Crude calcium cyanide produced by fusion of calcium cyanamide and common salt was first produced in commercial amounts in 1917; its subsequent development has been so rapid that by 1923 it supplied more than half of the world's cyanogen requirements. It appears on the market in lustrous black flakes, about 1 mm. thick, and of irregular area. It is sold on a basis of its contained cyanogen. An average analysis of the material now on the market is as follows.—

CN^-	= 25.44% equivalent to 45.03% $\text{Ca}(\text{CN})_2$ or 47.94% NaCN .
Cl^-	19.79
Ca^{++}	32.13
Na^+	12.11
CN_2^-	0.88
SiO_2	0.87
R_2O_3	1.95
Free carbon	= 2.81
CaC_2	1.90

The calcium cyanide prepared from calcium cyanamide has found wide application in the mining industry, where its lower cost enables it to compete successfully with pure sodium cyanide, in spite of its lower cyanogen content. It is also used in large amounts as a raw material for the manufacture of hydrocyanic acid, of prussiates, and of a number of other chemical products. In contact with moist air calcium cyanide liberates its cyanogen as free hydrocyanic

acid. This property is being utilised commercially. Quayle (*J. Econ. Entomol.*, 1923, **16**, 327) discusses its application as a fumigant for citrus scale, as a soil fumigant, and as a larvacide for control of the peach borer. Flint (*J. Econ. Entomol.*, 1923, **16**, 328) describes further developments in the same field.

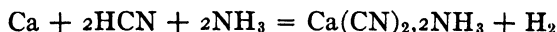
An interesting process for the preparation of high-grade calcium cyanide has been described by Metzger (*Ind. Eng. Chem.*, 1926, **18**, 161). Liquid hydrocyanic acid is added to finely ground calcium carbide. The following reaction takes place—



This is a light brown solid, very finely divided, which contains about 55% CN. It emits its hydrocyanic acid rapidly in contact with moist air and is recommended for use in fumigation. To this material Metzger has given the name "Powdered HCN."

Franck and Freitag (*Z. angew. Chem.*, 1926, **39**, 1430) have repeated the experiments of Metzger. In agreement with Metzger, they have found that the compound produced by the action of hydrocyanic acid upon calcium carbide is not homogeneous, but consists of two separately attached CN ions, HCN and $\text{Ca}(\text{CN})_2$. The molecular hydrocyanic acid is carried off by a current of indifferent gas, rapidly at first, then less rapidly, the compound approaching a composition corresponding to half the original content of CN. It is concluded that Metzger's compound contains about 55% calcium cyanide and about 25% of hydrocyanic acid. Since calcium carbide, the raw material for this synthesis, is not obtainable in a pure form, it has not been found possible to produce pure calcium cyanide in this way.

For the production of pure calcium cyanide Franck and Freitag dissolved anhydrous hydrocyanic acid in anhydrous ammonia and then added chips of metallic calcium. The di-ammoniate of calcium cyanide is formed according to the reaction—



This di-ammoniate is a white powder which can be kept unchanged in absence of air. When heated for two hours in a vacuum at 180° all the ammonia was evolved and pure calcium cyanide obtained. Materials prepared in this way contained up to 99% of pure calcium cyanide.

In analysing calcium cyanide, particularly in preparing the sample for analysis, it must be kept in mind that the material must be protected from exposure to moist air, since otherwise hydrocyanic acid will be lost. For this reason it is recommended that a large sample be taken for analysis, so that grinding of the sample may be omitted.

Weigh 20 grm. of the sample, and immediately transfer it to an ordinary $2\frac{1}{2}$ litre bottle containing $1\frac{1}{2}$ litre of water. Without stoppering the bottle give its contents a whirling motion and continue this at frequent intervals for two minutes. Then allow the bottle to stand for one hour with occasional stirring. Decant the solution into a 2,000 c.c. volumetric flask. Wash any undissolved residue into a porcelain mortar and grind, finally transferring it to the volumetric flask. Add 10 c.c. of a 15% solution of lead acetate, and make up to volume. After thorough shaking pour through a dry paper and pipette out 50 c.c. of the solution, equivalent to 0.50 grm. of the original sample. Dilute to 300 c.c. and titrate with silver nitrate, using potassium iodide as indicator.

Barium Cyanide.—This is the most stable of the alkaline earth cyanides. Hydrated barium cyanide, $\text{Ba}(\text{CN})_2 \cdot 2\text{H}_2\text{O}$, crystals lose one molecule of water by being dried *in vacuo* over sulphuric acid. The second molecule can be driven off in a current of air at 75 to 100° .

Barium cyanide may be produced in solution by absorption of hydrocyanic acid in barium hydroxide. It is also produced in the dry way by the action of atmospheric nitrogen on barium carbonate or oxide in presence of carbon. This operation was formerly conducted on a large scale by the procedure of Margueritte and Sourdeval (*Compt. rend.*, 1860, **50**, 1100). The process has long been abandoned, but there have been several recent attempts to adapt the reaction to modern conditions. Askenasy and Grude (*Z. Elektrochem.*, 1922, **28**, 130) give a comprehensive bibliography of the fixation of nitrogen by the barium cyanide process.

Copper Cyanide.—When a solution of an alkali cyanide is added to a cupric salt solution a yellow precipitate is formed which may be cupric cyanide. This compound is unstable and gives off cyanogen and is converted into cupro-cupric cyanides of variable composition. The copper cyanide of commerce is the cuprous salt, which is obtained as a white curdy precipitate by the addition

of a cuprous salt to a solution of an alkali cyanide or an alkaline earth cyanide.

Cuprous cyanide is an exceedingly stable compound. It is insoluble in water and is unattacked by hydrogen sulphide and by sulphuric acid. It is slightly soluble in ammonia and in solutions of ammonium salts. By boiling with dilute hydrochloric acid the cyanogen is quantitatively liberated as hydrocyanic acid. Nitric acid completely decomposes it.

Cuprous cyanide is readily soluble in alkali cyanides, and such solutions find wide application in the electro-plating industry. Commercial copper cyanide is seldom pure white, the tint being usually light yellow, with an occasional suggestion of green or blue. It is sold on guarantee to contain 70% copper (theory, 70.96% Cu). It is usually very pure, the cyanogen content corresponding closely to the requirement for CuCN . The cyanogen content of five samples of commercial copper cyanide produced by three manufacturers was found to be 28.71%, as compared with the theoretical percentage of 29.04.

Copper cyanide is analysed for copper by digestion with nitric acid and titration by the usual methods of inorganic analysis, preferably by the iodide-thiosulphate method. The cyanogen content of copper cyanide is best determined by evolution and absorption. Weigh a 0.5 grm. sample into a round-bottomed distilling flask, add 200 c.c. of water and 5 grm. of sodium chloride, and connect the side-arm of the flask with a small condenser dipping under the surface of 100 c.c. of 2% caustic soda solution. Add to the contents of the distilling flask through a dropping funnel 10 c.c. of 1:1 sulphuric acid, heat to boiling, and boil until the liquid in the distilling flask is reduced one-half. A little methyl orange indicator should be added to the absorbing solution as an indicator that sufficient hydrochloric acid has not distilled over into the absorbing solution to make it acid. Titrate the distillate with silver nitrate, using potassium iodide as indicator.

Zinc Cyanide.—Zinc cyanide is obtained by addition of a soluble zinc salt to a solution of sodium or calcium cyanide. It is a white compound insoluble in water and alcohol. It is soluble in alkaline cyanide solutions.

Zinc cyanide is an important article of commerce, used extensively in the electro-plating industry for zinc plating of certain

special articles, and, in conjunction with copper cyanide, for the electro-deposition of brass. The commercial compound should be pure white; it is usually sold under guarantee to contain at least 55% of zinc (theory, 55.68% Zn). Although the zinc content is thus considered an indication of the purity of the product, it is, as a matter of fact, of but slight value for this purpose, since all commercial zinc cyanide contains less cyanogen than the equivalent of the total zinc, the excess being present as oxide or hydroxide, and carbonate. The theoretical CN content of zinc cyanide is 44.32%. No commercial samples examined by the writer contained this percentage of cyanogen, the values found varying between 36.15% and 42.05%. In view of the fact that the cyanogen component is more expensive than the zinc component, some attention should be given by purchasers to the content of the former.

The cyanogen component of zinc cyanide is most conveniently estimated by evolution and absorption. The method employed is exactly the same as that described for copper cyanide, except that the addition of sodium chloride to the contents of the distilling flask may be omitted.

Silver Cyanide.—Silver cyanide, AgCN , is prepared by precipitation from a silver nitrate solution with hydrocyanic acid or alkali cyanide. It forms a white, cheesy precipitate, differing from silver chloride in that it is not affected by light. Silver cyanide is insoluble in water and dilute acids, but is readily soluble in alkali cyanide and ammonia. Concentrated hydrochloric acid decomposes it into silver chloride and hydrocyanic acid, while hydrogen sulphide gives silver sulphide and hydrocyanic acid. When heated to $320\text{--}350^\circ$ it melts and decomposes to metallic silver and cyanogen. A part of the latter is converted into paracyanogen, which remains with the silver until the temperature has been raised somewhat above the original decomposition temperature. Silver cyanide finds a considerable application in the electro-plating industry. Commercial silver cyanide is guaranteed to contain at least 80% of metallic silver.

Gold Cyanide.—Gold cyanide exists as aurous cyanide, AuCN , and auric cyanide, $\text{Au}(\text{CN})_3$. Aurous cyanide is formed by heating potassium aurocyanide with hydrochloric acid. It forms a light yellow crystalline powder which is insoluble in water and mineral acids. It is, however, easily soluble in potassium cyanide and

ammonia. When aurous cyanide is ignited it forms metallic gold and cyanogen.

Mercuric Cyanide.—Mercuric cyanide, $\text{Hg}(\text{CN})_2$, is prepared by dissolving mercuric oxide in an excess of hydrocyanic acid. It crystallises in colourless quadratic prisms and is extremely poisonous.

Mercuric cyanide is one of the few simple cyanides of the heavy metals soluble in water. Owing to its stability it reacts in an anomalous manner. Thus it does not respond to the iron test for cyanides (page 493), and is not precipitated by silver nitrate. It yields, however, a yellowish-white precipitate of palladious cyanide on addition of palladious nitrate. Mercuric cyanide is not precipitated by alkalis, but by boiling with hydrochloric acid hydrocyanic acid is evolved and mercuric chloride formed. Solutions of mercuric cyanide are readily decomposed by hydrogen sulphide and, after separation from the precipitated mercuric sulphide, the cyanide in the liquid can readily be estimated by the Liebig method (page 504).

Owing to the tendency to form mercuric cyanide, many simple and double cyanides are decomposed by boiling with yellow mercuric oxide and water. This is true of ferrocyanides and ferricyanides, and also of Prussian blue, but not of the cobaltcyanides.

No mercurous cyanide is known; on adding mercurous nitrate to a liquid containing hydrocyanic acid, or a metallic cyanide, metallic mercury separates and soluble mercuric cyanide is formed. A similar reaction occurs on treating calomel with excess of hydrocyanic acid. When dry mercuric cyanide is heated it decomposes, with formation of metallic mercury, paracyanogen and cyanogen gas.

A hot solution of mercuric cyanide readily dissolves yellow mercuric oxide. The resulting solution has been recommended, instead of solid mercuric oxide, for the separation of cobalt from nickel.

Mercuric cyanide may be estimated by treatment with hydrogen sulphide as described above, or by decomposition with stannous chloride. In the latter case the mercuric cyanide, to which has been added about one grm. of sodium chloride, is dissolved in about 200 c.c. of water in a round-bottomed distilling flask. To this solution are added 50 c.c. of saturated aqueous tartaric acid and 20 c.c. of a 5% solution of stannous chloride. The mercuric cyanide is decomposed, with separation of metallic mercury, whilst the hydro-

cyanic acid is liberated and may be distilled off, absorbed in caustic soda, and titrated by Liebig's method.

Fabre and Jossett (*J. pharm. Chim.*, 1923, **28**, 61) discuss the toxicology of mercuric cyanide. Its toxicity is due to the hydrocyanic acid set free, and the symptoms are those of HCN poisoning. Although dilute acids alone do not attack mercuric cyanide readily, the action is rapid in presence of albuminous materials, such as blood and gastric juice.

THE DOUELE CYANIDES

As has been stated elsewhere, the cyanides of the heavy metals exhibit a remarkable tendency to form double salts, which, in many instances, are of an exceedingly stable nature, so that in some cases neither the cyanogen nor the heavy metal is recognisable by any reaction which does not involve actual destruction of the double cyanide.

Certain of the less stable double cyanides are decomposed on addition of a dilute mineral acid, as in the following instance:



Double cyanides which suffer decomposition in this manner respond to the tests for simple cyanides, except that the precipitate produced by addition of silver nitrate to a neutral solution of the double cyanide does not yield pure silver cyanide, but a mixture of two metallic cyanides, as follows:

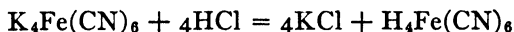


On treating the precipitate with dilute nitric acid the zinc cyanide dissolves and the silver cyanide remains.

Of the readily decomposable double cyanides, those of mercury, silver, zinc and cadmium are decomposed by hydrogen sulphide readily and completely, with precipitation of the corresponding metallic sulphide. Most of the other double cyanides of the class (*e. g.*, copper and nickel) are decomposed very incompletely, or not at all.

Other of the double cyanides are of a more stable character, and, on treatment with a dilute mineral acid, a new acid is liberated whose anion consists of a stable combination of hydrocyanic acid with a metal cyanide. Examples of such stable metallic cyanides

are the ferrocyanides and the cobalticyanides, which, on acidification, yield stable acids, hydroferrocyanic acid and hydrocobalticyanic acid, respectively. These acids give rise to a complete and characteristic series of salts.



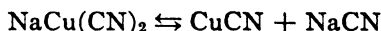
From the foregoing description it will be evident that the double cyanides may be conveniently arranged in two classes: (1) those which are readily decomposed by dilute mineral acids; and (2) those which are not materially affected by such treatment.

READILY DECOMPOSABLE DOUBLE CYANIDES

These compounds have a great practical interest from their application in the treatment of gold and silver ores and in electro-metallurgy. The more important compounds of the group are the following:

Double Copper Cyanides.—Cuprous cyanide dissolves readily and completely in the alkali and alkaline earth cyanides, to produce alkali or alkaline earth cuprocyanides. There are a series of these, of which the salts having the composition corresponding to $\text{NaCu}(\text{CN})_2$ are the most important. The ammonium salt of this series $\text{NH}_4\text{Cu}(\text{CN})_2$ is sparingly soluble in water; the potassium salt of the same ratio is moderately soluble and crystallises without water. The corresponding crystalline barium salt has the formula $\text{BaCu}_2(\text{CN})_4 \cdot \text{H}_2\text{O}$, and the calcium salt $\text{CaCu}_2(\text{CN})_4 \cdot 5\text{H}_2\text{O}$. According to Williams (*Chemistry of the Cyanogen Compounds* (1915), p. 50) three molecules of water are given off by the calcium salt at 100° .

The sodium salt is relatively insoluble and is very easily prepared by moderate evaporation of a solution of sodium cyanide in which copper cyanide has been dissolved, or from a solution of cuprous cyanide in calcium cyanide containing sodium chloride. It crystallises with two molecules of water. This double cyanide does not dissolve in water to a clear solution, but addition of a very small amount of alkali cyanide dissolves the precipitated cuprous salt and the solution becomes clear. Evidently an equilibrium exists, as indicated by the relation.



Other series of cuprocyanides are known in which the ratio between metallic cyanide and alkali cyanide is different from that

of the compounds just described. The sodium salt obtained by crystallising from a solution containing an excess of alkali cyanide has the formula $\text{Na}_2\text{Cu}(\text{CN})_{33}\text{H}_2\text{O}$.

Solutions of copper cyanide in sodium cyanide are not precipitated by alkali or by hydrogen sulphide, a fact utilised in analysis to separate copper from cadmium. In contact with iron they do not precipitate copper, and this behaviour is utilised in the electro-deposition of copper on iron surfaces. A solution of ammonium cupric sulfate becomes colourless or faintly yellow on addition of an alkali cyanide—a fact on which is based Parke's process for the volumetric estimation of copper.

Double Gold Cyanides.—Double salts of the alkalies and of trivalent gold may be produced by addition of auric chloride to strong alkali cyanide solution. The potassium salt has the formula $\text{KAu}(\text{CN})_{41}\frac{1}{2}\text{H}_2\text{O}$ (Williams, *loc. cit.*). The double salts of monovalent gold are, however, much more important in the arts. The aurocyanides are obtained by dissolving aurous cyanide or oxide in alkali cyanides or by solution of metallic gold itself in presence of oxygen. Auro-cyanides of most of the alkali and alkaline earth metals have been prepared. Williams gives their formulæ as follows:

Potassium salt	= $\text{KAu}(\text{CN})_2$
Ammonium salt	= $\text{NH}_4\text{Au}(\text{CN})_2$
Barium salt	= $\text{Ba}(\text{Au}(\text{CN})_2)_{2,2}\text{H}_2\text{O}$
Calcium salt	= $\text{Ca}(\text{Au}(\text{CN})_2)_{2,3}\text{H}_2\text{O}$
Cadmium salt	= $\text{Cd}(\text{Au}(\text{CN})_2)_2$
Sodium salt	= $\text{NaAu}(\text{CN})_2$

The application of cyanide to the extraction of gold from its ores is based upon the formation of such complex aurocyanides.

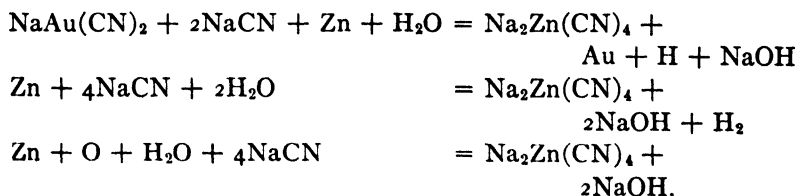
Double Silver Cyanides.—Compounds of the type $M^+\text{Ag}(\text{CN})_2$ are obtained by dissolving silver cyanide in alkali or alkaline earth cyanides. Such solutions are decomposed on addition of a mineral acid, with evolution of hydrocyanic acid and precipitation of silver cyanide. Salts of this type crystallise well and are stable. They are decomposed by treatment with hydrogen sulphide or ammonium sulphide. On the formation of such double compounds is based the extraction from its ores of silver by the cyanide process. Solutions of sodium or potassium silver cyanide are extensively used in electro-plating.

Double Zinc Cyanides.—Zinc cyanide dissolves readily in solutions of the alkali or alkaline earth cyanides, to form soluble double salts of the type $K_2Zn(CN)_4$. Potassium zinc cyanide is readily prepared by treating a solution of zinc sulphate or chloride with an equivalent amount of potassium cyanide and dissolving the washed precipitate of zinc cyanide in a solution of a second equivalent of potassium cyanide. On concentration, the solution deposits large colourless octahedra of the double cyanide, which crystallises without water. The salt is fusible, permanent in the air, and very soluble in water. Addition of a moderate amount of acetic, hydrochloric or sulphuric acid to the solution precipitates zinc cyanide, which dissolves in an excess of the reagent.

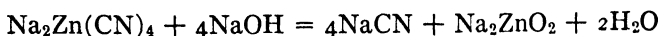
The corresponding sodium salt is very soluble and can be obtained by crystallisation only with great difficulty. It crystallises with three molecules of water. According to Herz (*J. Amer. Chem. Soc.*, 1914, 36, 912) one part of this hydrated salt dissolves in 0.47 part of water at 15°. The crystals are brilliant when removed from the solution, but effloresce rapidly, becoming dull and chalky. The dry salt is quite stable in air, being completely soluble in water after several days' exposure. Even after dehydration at 105°, there is little evidence of decomposition.

The barium salt crystallises easily with two molecules of water. The calcium salt is stable in solution, but, on account of its great solubility, cannot be obtained in crystalline form from aqueous solution.

The double cyanides of zinc are important constituents of the working solutions used for the extraction of the precious metals from their ores. They are, in part, formed by reaction with zinc minerals occurring in the ores, but are particularly due to reaction with the zinc dust or shavings which are used for the precipitation of the precious metals. Some of the reactions by which zinc enters these solutions are as follows:



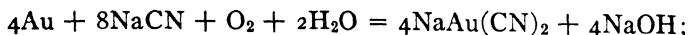
Whether the zinc double cyanide itself is a solvent for precious metals is open to question, but in actual practice its solutions are hydrolysed by the alkali of the solution, with formation of considerable amounts of alkali cyanide. The alkali cyanide so liberated is an active solvent for gold. The reaction of the hydrolysis is probably as follows:



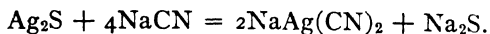
The dissolving power of a mill solution which has been in use for some time is thus partially dependent upon the alkalinity of the solutions; consequently an accurate control of solution alkalinity is essential for successful mill operation.

The Cyanide Process for the Extraction of the Precious Metals from Their Ores.—An extended discussion of the application of cyanide solutions to the extraction of the precious metals is outside the scope of this work. For such details the reader is referred to the following volumes: Julian and Smart: *Cyaniding Gold and Silver Ores* (1921); Clennell: *The Cyanide Handbook*, 1915; Hamilton: *Manual of Cyanidation*; McFarren: *Cyanide Practice*. The last-named offers an excellent bibliography. To insure a clear understanding of the subsequent paragraphs relating to the analysis of cyanide mill solutions the following paragraphs descriptive of the process are presented:

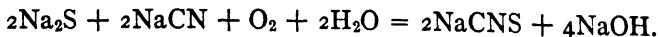
The solution of gold in cyanide is usually represented by the equation of Elsner:—



for the solution of silver occurring as sulphide the reaction is—



The sodium sulphide thus formed would prevent completion of the reaction, but is oxidised by the air, with formation, usually, of thiocyanate, thus:—



It is thus evident that oxygen plays a most important part in the extraction of the precious metals. Conversely, reducing conditions in such solutions are undesirable. Although in the great majority of cases atmospheric oxygen is the oxidising agent employed, there are occasional exceptions where a more active oxidising agent, *e. g.*,

sodium or barium peroxide or potassium permanganate has been employed with success.

The cyanide treatment may be applied to either coarse material, the sands, or to more finely ground material, the slimes. Formerly all cyanidation was performed on sands. The ground ore was in such cases classified and the slimes rejected, since their presence prevented penetration by the cyanide solutions. With the development of mechanical equipment for the treatment of slimes by agitation followed by sedimentation or filtration, it became general practice, after dividing the mill heads into sands and slimes, to treat them in separate circuits. Finally, with the introduction of modern equipment for fine grinding and with the perfecting of apparatus for the continuous treatment of slimes, the tendency has been towards all-sliming practice, all of the ore being finely ground and treated in an agitated circuit. In general, this has resulted in increased extraction, since many minerals are only slightly attacked, except when ground to impalpable fineness.

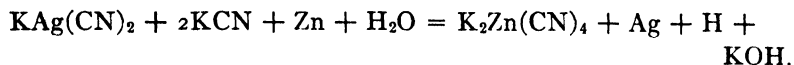
In all-sliming practice the ore may receive a pre-treatment, as for example, by grinding in water or in lime solution for the neutralisation of acidic constituents and for the elimination of the so-called cyanicides, or it may be ground in cyanide solution direct. Use of lime during grinding is general practice, its function being many-sided, namely, to neutralise the acidity of the ore, to prevent liberation of cyanide by atmospheric carbon dioxide, to regenerate cyanide from easily decomposable double cyanides, such as that of zinc, and to increase the settling rate of the mill pulps.

After grinding, the pulps are thickened to a density of from $1\frac{1}{2}$ parts to 2 parts of solution per part of solids, and the mixture is agitated with the strongest cyanide solution in the mill circuit for a period varying between 12 and 60 hours, the agitation being so conducted that an abundance of air is supplied to all parts of the mixtures. The concentration of cyanide solution used varies with the nature of the ore. For all-gold ores the concentration is from 0.005 to 0.05% NaCN; on silver-gold or all-silver ores it is higher, in special circumstances being carried as high as 0.5%. As stated on page 508, potassium cyanide was originally used exclusively. This was later displaced by the cheaper sodium cyanide, which in its turn has largely given place to the crude calcium cyanide described on page 511.

When solution of the "values" has taken place the pregnant solution is separated from the extracted ore by filtration, or by continuous counter-current decantation, either with or without a final filtration. The pregnant solution, after clarification, is ready for precipitation.

For the leaching of sands the ore is uniformly distributed by water into a series of tanks provided with false bottoms and containing layers of coconut matting, which act as filters. After filling with ore the water is drained away and is followed by addition of alkali cyanide solution of the maximum cyanide strength, say, 0.10 to 0.15% NaCN. This is allowed to stand on the ore for some hours, and is then drained off. This solution is followed by one or more weaker solutions and finally by water.

In either case, whether by leaching the sands or agitating the slimes, the gold or silver is usually recovered by precipitation on zinc. In exceptional cases carbon or aluminium may be used. In absence of gold, precipitation of silver may be effected by addition of an alkaline sulphide solution. In earlier practice the pregnant solution was passed through a series of boxes containing zinc shavings, but in the more modern mills the pregnant solution is mixed with an accurately determined quantity of metallic zinc dust, and the precipitated "values" are separated in a filter press. The chemistry of the precipitation reactions is obscure. Clennell represents the main precipitation reaction by the equation—



In addition to this main reaction, there are a number of side-reactions which also consume zinc. Zinc consumption is increased by the presence of dissolved oxygen in the precipitation liquors, and the modern development of apparatus for the deoxygenation of such solutions has greatly reduced zinc consumption.

The barren solution from the precipitation boxes or presses is fortified by the addition of fresh cyanide and is then returned to the process. The consumption of cyanide per ton of ore depends very largely upon the nature of the ore. On straight gold ores the consumption may be as low as 0.25 lb. NaCN per ton of ore and probably averages 0.4 lb. Losses of cyanide are due to a number of causes. Chemical losses are due to solution of iron, copper, or other base metals. Decomposition losses, resulting from the action

of acids or of atmospheric carbon dioxide, may occur where protective alkalinity is not carefully controlled. Losses by hydrolysis or by oxidation are also encountered. In addition to these chemical and decomposition losses, there are the mechanical losses of solution made necessary by the nature of the ore or by the plant flow sheet. These losses may constitute a very large part of the total loss, as in sand leaching, or they may be very small, as in slime plants employing filters.

The zinc precipitate from boxes or presses is fluxed for the recovery of the bullion. It may, or may not, be treated with acid to remove excess zinc before such treatment.

This brief discussion of the outstanding features of cyanidation will render intelligible the discussion of the analytical chemistry of mill solutions which follows:

Analysis of Cyanide Solutions in Mill Practice.—The methods of analysis used for the examination of the solutions in cyanide mill practice have been discussed in detail by J. E. Clennell (*The Cyanide Handbook*, 1910); the following is an epitome of this discussion:

The daily routine tests consist of: (1) estimation of free cyanide in the solutions entering and leaving the precipitation presses or boxes, or in the pumps and storage tanks, before and after making up to the required strength; (2) estimation of alkali hydroxides, the so-called protective alkalinity; and (3) assays of gold and silver in the solutions entering and leaving the precipitation presses.

In making the free cyanide test, solutions are frequently turbid from the presence of suspended matter. The solutions to be tested must be perfectly clear, and, if necessary, they must be filtered. Clarification with lime is rarely admissible, because it causes decomposition of the double zinc cyanides and thus raises the apparent free cyanide titration. Dilution, addition of alkali, variation of temperature and other conditions must also be avoided; all these affect the reading given by the silver nitrate, at least in the presence of zinc. The tests should be made in clean flasks, and the reaction observed in a good light against a dark background, as the first turbidity, which marks the end-point, is somewhat faint. From 10 to 50 c.c. of the solution, according to its strength in cyanide, are mixed with a few drops of a strong solution of potassium iodide and titrated with a solution of silver nitrate adjusted to give the

result with little or no calculation. Strengths commonly used are 13.04 grm. of AgNO_3 per litre or 17.33 grm. of AgNO_3 per litre; 1 c.c. of such solutions corresponds, respectively, to 0.01 grm. of KCN or 0.01 grm. of NaCN.

The end-point is usually taken at the first appearance of a permanent yellowish turbidity, a slight white cloudiness which may appear earlier being disregarded. This possibly gives slightly too high a result in presence of double zinc cyanides, as some of the cyanogen of these compounds may be also indicated when free alkali hydroxide is present. This is not, however, of much consequence if the test is made in the same manner in all solutions. In the absence of potassium iodide the end-point (white turbidity) is reached sooner, but is generally uncertain and indefinite. An intermediate result, and one which, so far as tested, appears to correspond with the actual working strength of the solution, is obtained by neutralising the free alkali hydroxide and monocarbonates, and then adding potassium iodide and titrating with silver nitrate in the usual way, taking as the end-point the appearance of a distinct permanent turbidity. The results may be calculated as per cent., or more often, as pounds of alkali cyanide, either sodium or potassium, per ton of solution.

In the absence of zinc the alkali hydroxide is estimated as follows: Silver nitrate is first added to a measured volume of the cyanide solution to be tested until a permanent turbidity is observed. Addition of a few drops in excess is immaterial. A drop of phenolphthalein solution is now added, and the liquid titrated with $N/10$ hydrochloric acid until the pink colour just disappears. The amount of the standard acid used measures the hydroxide content, the protective alkalinity, of the solutions. Results are calculated and reported as pounds of CaO per ton of solution.

In presence of zinc, before titrating with the acid add a sufficient excess (about 10 c.c.) of a 5% solution of potassium ferrocyanide and proceed exactly as above.

The total cyanide is defined by Clennell as "the equivalent in terms of potassium (or sodium) cyanide of all the cyanogen existing as simple cyanides and easily decomposable double cyanides, such as $\text{K}_2\text{Zn}(\text{CN})_4$." It is estimated by making the solution strongly alkaline with sodium hydroxide, adding potassium iodide, and titrating the cyanide with silver nitrate until a permanent yellowish

turbidity is observed. For 50 c.c. of the solution to be tested it is usually sufficient to add 10 c.c. of an indicator containing 4% NaOH and 1% KI.

The best method of estimating the total cyanogen present in a solution appears to be to boil with oxide of mercury in excess, filter, and remove mercury with an alkali sulphide. Any excess of sulphide is removed by agitating with lead carbonate, adding it in small quantities at a time and filtering. The clear liquid, after addition of potassium iodide, is titrated in the ordinary way with silver nitrate. By this method practically all of the cyanogen compounds are converted into mercuric cyanide, which is decomposed by the alkali sulphide into insoluble mercury sulphide and alkali cyanide.

In cases where all the iron in the solution exists in the form of ferrocyanide the latter is best estimated by determining the total iron, after decomposition of the cyanogen compounds. This may be effected by using a powerful oxidising agent, the iron then being precipitated by a slight excess of ammonia in the usual way.

Should much ferrocyanide be present, it is generally necessary to evaporate with nitric and sulphuric acids, sometimes more than once, until the liquid, made alkaline and re-acidified with hydrochloric acid, no longer shows a trace of blue colour. When this is the case the iron can then be separated by ammonia and estimated.

If insoluble iron compounds exist in suspension and cannot be removed by filtration, the solution should be agitated with lime and filtered, which will leave the soluble cyanogen compounds unaffected. If, however, the suspended matter itself contains insoluble ferrocyanogen compounds, they will be wholly or partly decomposed and a further quantity of ferrocyanogen added to the solution. In many cases Donath and Margosches' method (*Z. angew. Chem.*, 1899, 345) may be used to advantage. It is applicable for estimating ferrocyanide when iron is present in other forms and depends on the solubility of ferrocyanides in sodium hydroxide. The substance is first digested with 8% of sodium hydroxide until as much as possible is dissolved by gently warming. It is then filtered, and the filtrate is treated with brominised sodium hydroxide, which is prepared by dissolving 20 c.c. of bromine in a little of the 8% sodium hydroxide. A precipitate of ferric hydroxide is thus obtained, representing only that part of the iron which was originally present as ferrocyanide.

The ferric hydroxide is filtered off, preferably dissolved in hydrochloric acid, and reprecipitated with ammonia.

Ammonium thiocyanate, which may render the end-reaction in the silver nitrate titration somewhat obscure, is estimated by a colorimetric test. A portion of the filtered solution is acidified with hydrochloric acid, an excess of ferric chloride solution is then added, and the mixture is shaken up with a small quantity of ether; the same amount of hydrochloric acid, ferric chloride solution and ether are brought to a like volume by the addition of distilled water, and *N*/10 potassium thiocyanate solution is added until the red tint is the same as the tint formed by the solution under examination.

Determination of Metals in Cyanide Solutions.—All cyanogen compounds without exception (including ferrocyanides, ferricyanides and cobalticyanides) are completely decomposed, and the metals converted into sulphates or oxides by treatment in platinum with a mixture of three parts of concentrated sulphuric acid and one part of water. By heating the mixture until nearly all the acid is expelled, the residual mass will be obtained free from cyanogen. It may be dissolved in water or acid, and the metals estimated by the usual methods. This method is not adopted when mercury is to be determined, because some of this metal may be volatilised.

A standard method for the determination of gold is that of evaporation with litharge. A measured quantity of the solution, usually not more than 300 c.c. is placed in a porcelain dish, 20 to 50 grm. of litharge are then sprinkled over the surface of the liquid, and the mixture is allowed to evaporate at a gentle heat, without boiling. Towards the end of the evaporation care must be taken not to over-heat the residue, since this might cause it to adhere too firmly.

When quite dry, the residue is scraped out with a clean spatula and mixed with a suitable flux which may be varied according to circumstances. The following may be given as examples.

	(a)	(b)
Litharge (before evaporation).....	30	45
Borax.....	20	10
Sodium carbonate.....	16	40
Silica.....	25
Charcoal.....	0.5	1

The flux containing the dry residue is now transferred to a clay crucible and fused in an ordinary assay furnace. The charge is very fusible, giving a transparent nearly colourless slag, but in

some cases the slag may be stained, owing to the presence of iron, copper, and other impurities in the solution. The resulting lead button is then cupelled, and the bead of gold and silver parted in the usual way. This method, although somewhat long, is very reliable.

Another method in common use for the determination of precious metals in cyanide solutions is known as the Chiddy method. The procedure is as follows: Place five to ten assay tons of the solution in a beaker and heat almost to boiling. Add before or during the heating 10 c.c. of a clear saturated solution of lead acetate and 0.5 gm. of zinc dust. Stir well and heat nearly to boiling. Continue to heat for several minutes, and then add, with stirring, 15 c.c. of hydrochloric acid, and continue the heating. When the effervescence has ceased add more hydrochloric acid until the absence of action shows that the zinc is dissolved and the acid is in excess. The lead has now settled into a sponge, which should be tapped together and pressed into a mass with a glass rod to remove the water. Place the sponge on a piece of lead foil, $1\frac{1}{2}$ inches square, fold the foil loosely, and place it in a hot cupel for cupellation.

A satisfactory method for the determination of gold in electro-gilding solution consists in concentrating a measured quantity of the gilding solution in a porcelain dish. When a syrupy consistence is obtained a few gm. of pure red lead or litharge are added, the evaporation being then continued to complete dryness. The crucible is covered and brought for a short time to a moderate red heat. The lead oxide is reduced by the cyanide present, forming metallic lead and alkali cyanate, the reduced metal uniting with the gold and forming a button, in which the gold is determined by cupellation. The precious metals found in an electro-depositing liquid are generally reported in Troy ounces, pennyweights, and grains per pint of solution.

Silver is determined by the methods as described under Gold. In dilute solutions the silver may be separated, free from gold, by precipitating with sodium sulphide after adding a few drops of a solution of a lead salt. The precipitate should be washed with a dilute solution of sodium sulphide, dried, scorified and cupelled, or it may be converted into bromide by the addition of bromine, washed, dried, fused and weighed as silver bromide.

A method for the estimation of metallic silver in electro-plating solutions consists in taking a measured quantity of the liquid,

heating it to boiling, and then passing hydrogen sulphide through it, or else adding ammonium sulphide gradually. The silver, which is precipitated as silver sulphide, is liable to be contaminated with zinc and copper.

The precipitate is filtered off, washed, and finally washed into a flask or beaker and treated with an excess of bromine water. It is thus rapidly and completely converted into silver bromide. If any sulphur appears to have separated, a few drops of bromine should be added to ensure complete oxidation. The contents of the flask are diluted with water and boiled. The precipitate is now filtered off, washed, dried and weighed.

For the estimation of zinc and copper the usual methods of inorganic analysis are applied to a solution in which cyanogen compounds have been completely decomposed by evaporation with sulphuric acid.

It is frequently necessary to ascertain the "solvent activity" of a cyanide solution, *i. e.*, the efficiency of a cyanide solution for dissolving gold. The most satisfactory method (J. E. Clennell, *Eng. and Min. J.*, 1904, **77**, 513) is to prepare equal quantities of precipitated gold in a number of separate and similar vessels by means of gold chloride and sulphuric acid, making them faintly alkaline with sodium hydroxide, and adding to each a fixed volume of the various solutions to be compared. After being agitated for a definite length of time the residual gold is filtered off, dried, cupelled and weighed. The difference between this weight and that of the gold taken is a measure of the solvent activity of the solution.

DIFFICULTLY DECOMPOSABLE DOUBLE CYANIDES

In the preceding section we have considered those double cyanides which are decomposed by dilute mineral acids. We now turn to the other important group, which includes those double cyanides which are stable in the presence of dilute mineral acids. Of this latter group, the most important are the iron cyanogen compounds.

Compounds of Cyanogen and Iron.—Iron forms two important groups of compounds with cyanogen: ferrous cyanogen compounds and ferric cyanogen compounds. Of the simple iron cyanides, only ferrous cyanide, $\text{Fe}(\text{CN})_2$, is known. It is produced from a ferrous sulphate solution by precipitation by an alkali cyanide solution. It is a white amorphous product which easily oxidises in the air to a

blue colour. It is easily soluble in alkali hydroxide and carbonate solutions, forming characteristic double cyanides which are distinguished from the double cyanides previously described by their extraordinary stability. Neither the iron nor the cyanogen can be detected in these double cyanides by the usual tests, a complex and exceedingly stable anion having been formed, corresponding to the formula $\text{Fe}(\text{CN})_6^{4-}$. This anion is so stable that concentrated hydrochloric acid will not destroy it. Metallic salts containing the anion are designated ferrocyanides, and the hydrogen compound is the stable acid, hydroferrocyanic acid.

By oxidising the ferrocyanides with chlorine, bromine or other strong oxidising agent the iron component of the anion is oxidised to the trivalent form, and the resulting complex is the ferricyanide ion. Here, also, the iron and the cyanogen are so firmly bonded that the compound does not respond to tests for either iron or cyanogen. Reducing agents easily convert ferricyanide into ferrocyanide. The alkali ferro- and ferricyanides are easily soluble in water; of the alkaline earth salts, the calcium salts are readily soluble while those of barium and of magnesium are moderately soluble. Other metallic ferro- and ferricyanides are insoluble in water, and many of these are strongly coloured.

The Ferrocyanides

The ferrocyanides were originally the most important of the cyanogen compounds. The readiness with which they were formed, their stability, and the convenience with which their salts could be recovered in a pure form, made them valuable as means for the separation of cyanogen from mixtures. They were originally the starting material for the manufacture of nearly all of the other cyanogen compounds. Ferrocyanides are present in considerable proportion in the spent oxide and ammoniacal liquor of the gas works, and in former years all the ferrocyanide compounds were so obtained. As has been related elsewhere, the ferrocyanides were also formerly used in large quantity for the manufacture of the alkali cyanides.

Hydroferrocyanic Acid.—The free acid, hydroferrocyanic acid, may be obtained by adding to a 50% solution of potassium ferrocyanide an equal weight of strong hydrochloric acid and a little ether. The free acid is precipitated and may be recovered

by filtration on a Buchner funnel and washing with hydrochloric acid. The crude acid is then dissolved in alcohol, filtered, and the acid reprecipitated by means of ether. For complete removal of potassium chloride the acid is washed with absolute alcohol.

Hydroferrocyanic acid has strongly acidic properties, combining with all bases to form the corresponding salt. The solubility of the acid in water is 15 parts in 100 of water at 14° (Joannis, *Ann. Chim. Phys.*, [5], 1882, 26, 514). The acid is soluble in alcohol and dissolves without decomposition in concentrated sulphuric acid. It is a strong acid, acting readily on carbonates and on salts of many organic acids. The dry acid is stable up to 100°, but if heated much above this temperature it begins to decompose. The acid heated in water decomposes, forming hydrocyanic acid and ferrous cyanide. The ferrous cyanide so formed is stable up to about 450°, when it gradually breaks up, forming iron carbide and nitrogen.

Calcium Ferrocyanide.—Calcium ferrocyanide is prepared commercially by addition of a solution of copperas to a solution of calcium cyanide, or by conversion of calcium ammonium ferrocyanide by boiling with an excess of lime. It is exceedingly soluble in water, but may be obtained in crystalline form containing eleven molecules of water (Farrow, (*J. Chem. Soc.*, 1926, 129, 49). A limited amount of calcium ferrocyanide is used in the industries.

More interesting are the double calcium ferrocyanides of potassium and ammonium. Both are very insoluble, and by their production a simple method is provided for obtaining the ferrocyanides free from foreign salts. Ammonium calcium ferrocyanide is easily obtained by addition of an ammonium salt to a ferrocyanide solution containing calcium. The double salt separates in small white crystals which are easily separated from the mother liquor. By boiling with lime the ammonia is displaced and a pure solution of calcium ferrocyanide is obtained.

The double potassium calcium ferrocyanide is even more important. This salt is produced as an intermediate product in most processes for the production or purification of potassium ferrocyanide. By addition of potassium chloride to a solution of calcium ferrocyanide or of calcium chloride to a solution of potassium ferrocyanide the double salt is produced in small anhydrous crystals which are only slightly soluble in water. At 15° 100 c.c. of water

dissolve 0.35 grm. of the ammonium salt and 0.72 grm. of the potassium salt. The compounds are not appreciably more soluble in hot water.

Heavy Metal Ferrocyanides.—Although few of these compounds are of importance to the industrial chemist, several of them have interest for the analytical chemist, owing to the fact that soluble ferrocyanide solutions are frequently used for the identification and estimation of the metals. Thus solutions of ferrocyanide are very commonly employed for the detection of small amounts of copper, this reagent being one of the most sensitive to this metal. In this test cupric ferrocyanide separates as a red or red brown gelatinous precipitate, the colour and appearance varying somewhat with the conditions of the precipitation.

Lead, cadmium and zinc ferrocyanides are white and all are familiar to the analytical chemist. The composition of the precipitates obtained when ferrocyanide solutions are employed for the volumetric determination of the heavy metals depends upon the reaction of the solution and upon the alkali cations present. It is well known to chemists employing the ferrocyanide titration for the determination of zinc that the zinc value of a given ferrocyanide solution is different in the acid and in the alkaline titration, both of which are standard analytical methods. Treadwell (*Helv. Chim. Acta*, 1922, **5**, 633) has shown that when zinc is titrated electrometrically in neutral solution with sodium ferrocyanide the precipitate is $\text{Zn}_2\text{Fe}(\text{CN})_6$, while with potassium ferrocyanide the end-point is reached when all the zinc is precipitated as $\text{K}_2\text{Zn}_3(\text{Fe}(\text{CN})_6)_2$. The composition of the precipitate in the latter case is unaffected by the presence of acids. This latter compound is also secured when the zinc salt is mixed with potassium chloride and hydrochloric acid before titrating with sodium ferrocyanide. When a cadmium salt is titrated with potassium ferrocyanide a precipitate is obtained corresponding to $\text{K}_2\text{CFe}(\text{Cn})_6$ only in very dilute solutions. At higher concentrations the precipitate is richer in cadmium. When sodium ferrocyanide is used the precipitate is $\text{Cd}_2\text{Fe}(\text{CN})_6$. In titrating lead with potassium ferrocyanide the precipitate is $\text{Pb}_2\text{Fe}(\text{CN})_6$. This variation in composition makes it essential to standardise the ferrocyanide solutions which are to be used for titration under as nearly as possible the exact conditions under which the regular analysis will be made.

Potassium Ferrocyanide.—The potassium salt was formerly the industrially important ferrocyanide, but it has been almost entirely displaced in manufacturing processes by the cheaper sodium salt, so that today the consumption, particularly in the United States, is very small.

Potassium ferrocyanide is produced commercially by separation of insoluble calcium potassium ferrocyanide from a solution of any convenient ferrocyanide, and conversion of the precipitate into soluble potassium ferrocyanide by boiling it with potassium carbonate. Its crystals are amber-yellow in colour, and contain three molecules of water. They do not lose water of crystallisation in air of ordinary temperature and humidity, differing in this respect from the sodium salt. Potassium ferrocyanide is not poisonous, but reacts on the human body as a purgative. Its solutions are moderately stable and are neutral in reaction. When heated in air potassium cyanate and iron oxide are formed, while potassium cyanide, iron carbide and nitrogen are formed when it is ignited in a closed vessel.

Potassium ferrocyanide is used in the manufacture of blues, although, as indicated above, it has been very generally superseded in this field by sodium ferrocyanide. It is still employed, however, for certain potash blues. It is also produced for conversion into potassium ferricyanide, and still smaller amounts find their way into the miscellaneous chemical field.

Sodium Ferrocyanide.—Sodium ferrocyanide, at present the most important of the ferrocyanides, crystallises in large well-formed crystals containing ten molecules of water. It effloresces in ordinary air, and when dried at 100° becomes entirely anhydrous.

Until quite recently the coal gas industry furnished the whole of the ferrocyanide salts used throughout the world. This industry is discussed in a later section entitled Cyanogen Compounds in Coal Gas (page 540). At the present time a substantial part of the world's ferrocyanide requirement is produced from crude calcium cyanide, and the recovery of the cyanogen compounds from coal gas has become unprofitable. This change in economic relations is of interest in showing the change introduced into the cyanogen industry by the development of the cyanamide process for the manufacture of alkaline earth cyanide. Heretofore the efforts of chemists were centered on the conversion of ferrocyanide into cyanide;

today, the largest producer of ferrocyanide compounds in the world employs cyanide exclusively as his raw material.

The solubilities of potassium, of sodium and of calcium ferrocyanides have been determined by Farrow (*loc. cit.*) as follows. The data record the grm. of anhydrous salt in 100 grm. of solution and the density of the solutions.

Temp.	$K_4Fe(Cn)_6$		$Na_4Fe(Cn)_6$		$Ca_2Fe(Cn)_6$	
	Grm.	d.	Grm.	d.	Grm.	d.
24.9°	23.95	1.1731	17.11	1.1312	36.44	1.3563
34.9	28.01	1.2018	20.58	1.1584	39.22	1.3662
49.8	33.13	1.2350	26.20	1.2004	42.04	1.3970
64.7	36.94	1.2635	31.43	1.2426	44.44	1.4074
79.6	40.45	1.2854	36.85	1.2786
84.7	38.15	1.2916
89.6	38.08	1.2959
94.7	38.25	1.2870
99.7	43.78	1.3115	37.53	1.2861

Ferrocyanides of Iron—The Ferro Blues.—Approximately 75% of the ferrocyanide salts produced and sold in the world are used in the production of the ferrocyanides of iron, which under the various names—Prussian Blue, Milori Blue, or Chinese Blue—are so widely used in the colour industry. The term Prussian Blue is frequently used as a generic term applicable to the iron blues in general, but since in the trade Prussian Blue has reference to a blue of particular shade, it will be preferable to employ the generic term ferro-blues or iron blues to distinguish the iron ferrocyanide blues from other blues, for example from ultramarine blue. In view of their great importance in industry, the iron blues merit a somewhat extensive discussion.

When a solution of a ferric salt is added to a neutral or acid solution of an alkali ferrocyanide a blue precipitate is produced, the composition and properties of which depend upon the conditions of the precipitation and upon the alkali ferrocyanide used. This is a familiar analytical test for the presence of ferrocyanide, and the blue precipitate so obtained is frequently termed Prussian blue; however, as will be shown below, the Prussian blue of commerce

is made in an altogether different way, and its composition is different from the composition of the precipitate obtained in the "Prussian blue test."

Although the ferro-blues are conveniently represented as ferrocyanides of ferric iron, they are, in reality, compounds of a much more complicated nature. All of them contain alkali as a constituent of the molecule, and the characteristics of the compound depend upon the nature and amount of the alkali contained in them, as well as upon the manner in which the precipitation has been conducted. The iron blues were originally manufactured exclusively from potassium ferrocyanide, and it was considered impossible to produce a satisfactory colour with sodium ferrocyanide. Ammonium ferrocyanide, however, yields a blue which closely resembles the blue obtained from potassium ferrocyanide, and it has now been found possible to produce blues of quality comparable with the potash blues by the addition of ammonium sulphate to sodium ferrocyanide. The success of these soda or soda-ammonia blues is responsible for the great diminution in the quantity of potassium ferrocyanide now consumed by the colour trades. Potash blues are now the exception, although they have certain advantages in quality over the soda blues, in that they have, as a rule, a greater depth of tone and greater warmth. In the case of the bronze blues especially, the superior lustre or bronziness of the potash blues is particularly noticeable when used in printing inks.

In addition to this combined or adsorbed alkali, the ferro-blues contain a considerable amount of water of constitution, much of which is retained even on heating to high temperature. Thus a particular sample of blue containing 10.13% of total water lost only 5.92% when heated *in vacuo* at 90°. Complete removal of this water could only be accomplished by decomposing the blue.

Properties of Pigments.—In the valuation of blues, as in the valuation of most other pigments, chemical analysis casts but little light upon the properties and value of the material under examination. Valuation is based almost entirely upon the results of certain empirical physical tests which have been developed with particular reference to the purpose to which the material is to be put. Since the nomenclature of such test work may not be familiar to the analytical chemist, a few definitions will be instructive.

The *strength of a colour* is its ability to colour a maximum quantity of white pigment, or alternatively, its ability to colour a fixed amount of white pigment to a maximum tint.

The *oil absorption of a pigment* is a relative term denoting the amount of medium (oil or varnish) required to grind a fixed quantity of the pigment to a working paste.

The *softness of a pigment* is the ability of the pigment to mix with the medium with a minimum amount of grinding.

Purity of tone has reference to clearness of the shade and absence of a dirty or grayish hue.

The *working properties* of a paste or ink made by grinding the pigment with varnish have to do with its ability to work well on printing rollers, to lie well on the paper to be printed, and to be smoothly and evenly applied.

The *true colour* of a blue pigment is that colour observed by looking down on a print made from the pigment ground in a suitable vehicle, and viewing it by transmitted light.

The *overtone* or *top tone* is that shade seen when looking at a print by reflected light.

The *undertone* is that shade seen when a print is "pulled out," that is, printed very thinly, or when the colour is in reduction.

A pigment is said to be *in reduction* when it is incorporated in a medium with a white pigment. Blues are usually reduced with zinc oxide or with white lead in the proportion of 100 to 1, or occasionally 20 to 1.

All blues, whatever their specific properties, should be uniform in shade in the various deliveries of the product, should be fine, soft, free from grit, have low oil absorption and possess in maximum degree the properties of strength, brilliancy and purity of tone.

Varieties of Ferro-blues.—Having thus defined our terms and indicated the special properties in which the user of blue pigments has interest, we next turn to a description of the more important varieties of iron blues on the market and to their uses.

Bronze blue.—This is a variety of blue used extensively in lithographic printing work, the lithographic printing ink being formed by grinding the blue with a lithographic varnish. A peculiarity of this variety of blue is that it exhibits a high bronze effect when printed out, the less the varnish used the higher being the bronze.

One of the most valuable properties in a bronze blue is a low oil absorption.

Soluble blue is a variety of the product which is completely soluble in water. It is used in paper pulp, in laundry blue, for making writing inks, and in the pigment chrome green by mixing the blue solution with chrome yellow. Properly prepared soluble blue does not settle out on standing.

Chinese blue is extensively used in the paint trade, ground, as a rule, with a white base pigment such as zinc or white lead. Large quantities of Chinese blue are worked up with yellow (zinc and lead chromate) into various forms of chrome greens. When barytes is added they are frequently known as Brunswick greens. The blue may be sold as a dry powder or in pulp form. Shade cloth manufacturers use quantities of blue in this shade.

Milori blue is a very light coloured product of light gravity, with great strength, greenish undertone, and exhibiting little or no bronziness. It is used in typewriter ribbons and carbons and also occasionally where very clear greenish blues are required, as in distemper work. It is also used in three-colour printing.

Prussian blue or *blue for blacks* is very reddish in undertone, very blackish in top tone, and is used in the manufacture of patent leather varnishes. Both soda and potash Prussian blues are used for this purpose. It is also used in tinting black printing inks to make more intense and brilliant blacks, and in enamels and varnishes for black automobile bodies.

Manufacture of Ferro-blues.—The process of manufacture of the ferro-blues may be briefly summarised as follows. A solution of approximately 10% green copperas is run into an approximately 10% solution of sodium ferrocyanide to which ammonium sulphate, from 15 to 20% weight of the sodium ferrocyanide, has been added. The temperatures at which the solutions are run together and the time consumed in running them together, as well as the manner of stirring, are important factors in determining the nature of the final product. The white magma, which is a precipitate of ferrous ferrocyanide containing varying amounts of sodium and ammonia, is now acidified with either hydrochloric or sulphuric acid, and then an addition of an oxidising agent such as sodium chlorate or nitric acid is made. The batch is then maintained at a temperature varying from 60° to the boiling-point until the oxidation has pro-

ceeded to the proper point. The period of time over which the batch is oxidised and the violence of the oxidation are also determining factors in the final shade. The batch is next washed free from acids and soluble salts by decantation, and is then filtered, washed, dried and ground. This description applies to the Bronze, Chinese and Milori blues, the various colours being produced by variations in the technique.

Soluble blue is made from a good quality Chinese blue pulp by intimately mixing it with from 3 to 7% of oxalic acid (based on the dry weight of the pulp), or from sodium ferrocyanide by intimately mixing the pulp with a concentrated solution of the prussiate and subsequently stiffening the paste with starch or with an additional quantity of crystal sodium ferrocyanide.

Testing of Ferro-blues.—The following procedure is recommended for testing for *Top Tone, Oil Absorption, Working Qualities and Softness*.

1 grm. of the pigment is weighed out on a marble slab and sufficient lithographers' varnish added to enable the sample to be rubbed down to the consistence of a thick paste. The quantity of varnish required will vary from 0.8 to 1.2 grm. In the case of bronze blues it is usual to employ a thick varnish, such as No. 00; in the case of Milori or Chinese blue it is usual to employ a No. 1 varnish. The rubbing down is done with a glass muller and will require from 5 to 10 minutes. Comparison is made in all cases with a standard blue of known quality, and the treatment which the standard receives should be the same as that to which the unknown sample is subjected. The ease with which the blue is incorporated into the varnish to form the ink is a measure of its *softness*, and the quantity of varnish required to grind it to a working paste is a measure of the *oil absorption*.

The ink is then smeared in a thin film on the marble slab, and a small composition roller such as is used in lithographic work (consisting of a mixture of gelatin and glycerin) is rolled over the film of ink. The wet roller is then pressed lightly on a sheet of coated paper. This is repeated until there are several sheets with impressions of the ink. The resulting impressions are then compared with similar impressions made in the same way from the standard pigment.

When the impressions are dried they are carefully compared, and the experienced eye can readily detect any differences in tone or colour between the unknown and the standard. The ease with which the ink picks up on the roller and is laid down on the paper is a measure of the *working qualities* of the pigment.

Strength and undertone are determined by reducing 0.05 grm. of the blue with 5 grm. of zinc oxide. The two weighed powders are transferred to the marble slab, a definite quantity of pure linseed oil is added, and the whole mixed into a stiff paste with a spatula. Prolonged rubbing with a muller is then applied to bring out the full strength of the pigment. When the colour of the paste ceases to increase in depth the operation is completed, and the paste may then be placed on a glass slip and compared with a standard blue reduced alongside of it. The paste with the deeper colour, of course, contains the stronger pigment, and slight differences in shade and undertone become noticeable in the reduction that would not appear in a comparison of the unreduced colour.

Cyanogen Compounds in Coal Gas.—The gases resulting from the destructive distillation of coal gas were formerly the principal source of the cyanogen compounds of commerce and the exclusive source of the ferrocyanide compounds. Today the various synthetic cyanogen processes have far outstripped this earlier source, leaving it in a position of comparatively minor importance. In view of the fact that at present only the ferrocyanides are produced from coal gases, it will be in order to discuss at this point the recovery of cyanogen from coal gas and the production of ferrocyanides therefrom.

Coal contains from 1 to 2% of nitrogen, but of this only a very small part, 1 to 3%, is liberated as "cyanogen." In the coal gas industry the term "cyanogen" is applied to all the cyanogen compounds in the gas; for practical purposes it is equivalent to hydrocyanic acid, because all or nearly all of the cyanogen in the gas is present as hydrocyanic acid or as ammonium cyanide. This cyanogen is doubtless formed by a secondary reaction between the hot coke and the ammonia liberated during the distillation. The absolute amount of the cyanogen compounds occurring in coal gas is small, varying between 25 and 100 grains per 100 cubic feet.

In plants which have no special arrangements for the recovery of cyanogen a partial recovery is made in the iron oxide purifiers,

and as long as the cyanogen compounds absorbed therein could be recovered at a profit, there was no great objection to their accumulation in the purifying boxes. Under present conditions, however, the extraction of ferrocyanides from spent oxide is rarely profitable, and it is no longer good practice to depend upon the purifiers for cyanogen removal. Cyanogen recovery from spent oxide has been entirely abandoned in the United States and is carried on to a very limited extent in Europe.

The installation of special cyanogen washers for the removal from the crude gas of cyanogen compounds is considered desirable, not only to protect the iron oxide purifiers, but also because of the general belief that cyanogen removal reduces corrosion in the holders, meters and service mains. The older methods for this removal may be divided into two groups: those employing ferrous salts, and those employing polysulphides.

The largest installation for cyanogen recovery in the United States is at the Astoria plant of the Brooklyn Consolidated Gas Co., where the Bueb process, a leading representative of the first group, is employed. In this process the gas is passed through a scrubber to which a solution of ferrous sulphate is supplied. A series of reactions takes place in the scrubber between the ferrous salt solution and the impurities of the coal gas, cyanogen, ammonia and sulphur, which result in very complete recovery of the cyanogen as ferrocyanide.

By acidification of the scrubber effluent the whole of the cyanogen content of the mixture is precipitated as insoluble ferrous ammonium ferrocyanide and is recovered by filtration. The cyanogen sludge so produced is sold to another company and is converted into sodium ferrocyanide by boiling with lime, the ammonia component of the sludge being simultaneously recovered as ammonium sulphate.

A complete description of the process used at Astoria is given by M. E. Mueller (*J. of Gas Lighting*, 1910, 112, 851).

In the process of the British Cyanides Company, which is representative of the second group, the foul gas is passed through a bed of spent oxide kept moist by a water spray. The cyanogen is converted into thiocyanate and is delivered from the scrubber as a solution of ammonium thiocyanate. This process is simpler than the ferrocyanide processes, but recovers the cyanogen in a much less desirable form. For the utilisation of this thiocyanate solution it is first treated with sodium carbonate and the ammonia liberated and

recovered. The resulting solution of sodium thiocyanate is concentrated, mixed with finely divided iron, and finally boiled down to complete dryness and fusion. A reaction finally occurs, with formation of sodium cyanide and ferrousulphide. Digestion of the resulting mixture with water and filtration yields a solution of sodium ferrocyanide.

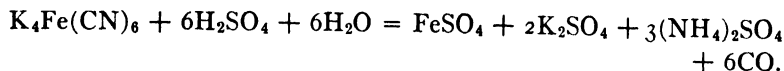
A recent development in cyanogen removal is the installation by several gas companies of the "Seaboard" process, in which cyanogen is removed simultaneously with sulphur by washing the gas with a solution of sodium carbonate (Sperr. *Tech. Sec. Amer. Gas Association*, 3rd Annual Convention, 1921, 3, 282). The removal of cyanogen by this process is satisfactory, but the sodium thiocyanate formed cannot be economically recovered.

The Detection and Estimation of Ferrocyanide

Detection.—Insoluble salts of hydroferrocyanic acid, when treated with alkali or alkali earth hydroxides, are converted into the corresponding alkali or alkaline earth ferrocyanides, which are soluble salts. To the solution so obtained the following tests may be applied.

Dilute sulphuric acid does not react with ferrocyanide solution except at temperatures near the boiling point, when a portion of the combined cyanogen is liberated as hydrocyanic acid.

Concentrated sulphuric acid reacts with ferrocyanides according to the following equation.



This reaction is frequently utilised for the production in the laboratory of carbon monoxide.

Most oxidising agents, chlorine, bromine, hydrogen peroxide, permanganate and other per-salts readily oxidise the ferrocyanides to ferricyanides.

Silver nitrate forms a white precipitate of silver ferrocyanide, insoluble in dilute nitric acid or ammonium hydroxide, but soluble in alkali cyanide solutions. If, however, it is oxidised with concentrated nitric acid to silver ferricyanide, an orange coloured precipitate occurs, which is readily soluble in ammonium hydroxide.

Soluble copper salts form a characteristic chocolate brown precipitate of cupric ferrocyanide. This is an exceedingly sensitive test for either copper or ferrocyanide.

Soluble ferric salts produce the Prussian blue test in neutral or acid solution of ferrocyanides. This is one of the most important qualitative tests for ferrocyanides. To apply the test to insoluble ferrocyanides they must first be heated with sodium hydroxide, the metal being precipitated as hydroxide and yielding sodium ferrocyanide. After removal of the metallic hydroxide by filtration the filtrate is made slightly acid and a solution of a ferric salt is added. A blue precipitate or a green coloration results, depending upon the quantity of ferrocyanide present. The precipitate is insoluble in dilute mineral acids, but dissolves in oxalic acid to a deep blue liquid, and in ammonium tartrate to a violet liquid. Like the other insoluble metallic ferrocyanides, it is decomposed by treatment with alkali hydroxides.

Estimation.—Four older and one recent method for the estimation of ferrocyanide merit description. These are as follows: (1) permanganate method, (2) titration with a copper salt, (3) titration with a zinc salt, (4) decomposition with a cuprous salt and titration of the liberated hydrocyanic acid, (5) precipitation of benzidine hydroferrocyanide.

Permanganate Method.—The permanganate method has the advantage of rapidity and the disadvantage of lack of specificity, as well as of an unsatisfactory end-point. In applying this method thiocyanates, sulphites, sulphides, thiosulphates and other reducing agents must be absent. In the absence of these impurities the titration is conducted as follows: A quantity of material containing about 1.0 grm. of the alkali ferrocyanide is dissolved in water, the solution diluted to about 700 c.c. and placed in a large porcelain casserole. The solution is acidified with dilute sulphuric acid and then standard potassium permanganate ($N/10$) is slowly added, with constant stirring, until the yellow colour of the solution changes to yellowish-red. The end of the reaction is not easy for the untrained eye, but with experience the titration is an accurate one. If a trace of ferric chloride is added to the liquid, the disappearance of the bluish-green colour will render the termination still more distinct. The reaction is

$$5\text{K}_4\text{Fe}(\text{CN})_6 + \text{KMnO}_4 + 4\text{H}_2\text{SO}_4 = 5\text{K}_3\text{Fe}(\text{CN})_6 + 3\text{K}_2\text{SO}_4 + \text{MnSO}_4 + 4\text{H}_2\text{O}.$$

The permanganate solution used should be standardised against potassium ferrocyanide or, preferably, against pure calcium potassium ferrocyanide, which can be produced in a very pure form and which crystallises without water. Preparation of this salt for standardisation is described below.

Copper Sulphate Method.—Williams (*Chemistry of the Cyanogen Compounds*, 1915, 352) discusses this important volumetric determination in great detail. The standard copper solution is prepared by dissolving 12.5 gm. of pure cupric sulphate in distilled water, adding 10 c.c. of 10% H_2SO_4 , and then diluting the whole to 1,000 c.c. This solution is standardised against calcium potassium ferrocyanide.

The double ferrocyanide for standardisation is prepared by adding a solution of calcium chloride to a moderately strong solution of pure potassium ferrocyanide. The double salt separates out in the cold in small granular crystals which are anhydrous. The crystals are filtered off, washed with distilled water, and dried at 100° . For use in standardisation one gm. of the salt is weighed out, converted into potassium ferrocyanide by boiling with a solution of potassium carbonate, filtering, washing the precipitated calcium carbonate free from soluble salts, and made to 100 c.c.

For analysis a 25 c.c. portion of the solution under examination is placed in a beaker of 150 c.c. capacity, acidified with 25 c.c. $N/5$ sulphuric acid, and 20 c.c. of saturated potassium chloride solution is added. The copper solution is now run in slowly from a burette, the liquor being agitated gently the while, until the whole of the ferrocyanide is precipitated. The end-point is observed by taking a strip of filter paper, about $1\frac{1}{2}$ inches long and $\frac{3}{4}$ inch wide, and dipping it into the liquid so that about $\frac{1}{3}$ is submerged. The strip is removed and the clear liquid which is sucked away from the brown precipitate of cupric ferrocyanide is touched with a glass rod that has been dipped in a dilute solution of ferric chloride. Any ferrocyanide still in solution will produce a blue coloration at the junction of the two liquids, care of course being taken that the iron salt does not touch the brown precipitate of copper ferrocyanide. This formation of a blue coloration indicates that ferrocyanide is still in solution and that more copper must be added. As the blue colour becomes less and less, smaller quantities of the copper solution should be added after each test. The final end-point is considered to be the point when no blue colour appears on the paper after the

lapse of one minute. Operations in the standardisation and in the analysis are to be conducted under exactly the same conditions.

Williams emphasises the importance of the following precautions:

1. The total volume of liquid titrated should be about the same in each operation, and the same as that used for the standard.
2. As nearly as possible the same amount of ferrocyanide should be present in each test.
3. It is essential that potassium salts should be in excess in the liquid and also, if caustic alkali has been used to decompose an insoluble ferrocyanide, this alkali must be caustic potash and not caustic soda.
4. When standardising the copper solution the warning given in No. 3 above must be observed, and an excess of potassium chloride or sulphate added before titration.

According to Williams the importance of the use of a potash salt in the solution to be titrated is due to the fact that the precipitate desired in this titration is a mixed ferrocyanide of copper and potassium, with the formula $\text{Cu}_6\text{K}_8(\text{Fe}(\text{CN})_6)_5$. By the addition of a suitable excess of potash solution either sodium or calcium ferrocyanide may be determined by this method, and the composition of the precipitate will be the same as that obtained by titration of potassium ferrocyanide.

Zinc Sulphate Method.—This procedure is one much used in control work in connection with the manufacture of cyanogen compounds. The analytical directions recommended by the Chemical Committee of the American Gas Association (*Gas Chemist's Handbook*, 2nd Ed., 1922, 323) are as follows:

The solutions required are:

1. Standard potassium ferrocyanide solution prepared by dissolving in distilled water 10 gm. of C.P. $\text{K}_4\text{Fe}(\text{CN})_6 \cdot 3\text{H}_2\text{O}$ containing the exact quantity of water of crystallisation and diluting to one litre.
2. Standard zinc sulphate solution prepared by dissolving in distilled water approximately 10.2 gm. of C.P. zinc sulphate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$), adding 10 c.c. of concentrated sulphuric acid, and diluting to one litre.
3. Solution of ferric alum indicator. Dissolve in distilled water 30 gm. of ferric alum, add 6 c.c. of concentrated sulphuric acid, and dilute to one litre.

4. Dilute sulphuric acid. A 10% solution.
5. Saturated solution of potassium sulphate.

Standardisation Procedure: Measure accurately into a 300 c.c. beaker 25 c.c. of the standard potassium ferrocyanide solution. Acidify with 10 c.c. of dilute H_2SO_4 and add 10 c.c. of saturated potassium sulphate. Dilute with sufficient distilled water to make the volume of the mixture about equal to that to be titrated when a sample is being evaluated. The amount of potassium ferrocyanide in the solution to be analysed should be approximately the same as the amount of potassium ferrocyanide contained in 25 c.c. of the standard ferrocyanide solution. If greatly different, a correspondingly larger or smaller volume of the standard ferrocyanide solution should be employed for the standardisation.

With constant stirring slowly run in the zinc sulphate solution from a burette until all of the ferrocyanide is precipitated. The end-point is observed by placing a drop of the mixture on a drop reaction or filter paper, and then placing a drop of the ferric alum solution near it, so that the two drops just touch each other upon absorption by the paper. The ferric alum solution must not touch the white precipitate in the centre of the drop taken from the beaker. Either Schleicher and Schülls' Drop Reaction Paper No. 601, or Baker and Adamson Grade A filter paper may be used. The particular kind of paper used for the standardisation of the zinc sulphate solution must be used in all subsequent titrations in which that same zinc sulphate solution participates. The test paper must be free from iron. A blue coloration at the point where the two drops meet indicates that all the ferrocyanide has not been precipitated. The end-point is reached when the blue coloration does not appear after an interval of one minute. Two portions of the ferrocyanide solution should be titrated. In the first portion the result between limits is obtained by adding the zinc sulphate solution, 0.5 c.c. at a time, as the end-point is being approached; in the second portion the exact end-point is obtained by adding the zinc sulphate to the point where the blue coloration was last seen in the previous titration and then continuing the addition, a drop at a time, until no blue coloration appears. At least two spot tests should be made at the end-point.

From the amount of the zinc sulphate solution required to precipitate the ferrocyanide from the given volume of standard potas-

sium ferrocyanide solution the value of the zinc sulphate solution is obtained.

1 c.c. $\text{K}_4\text{Fe}(\text{CN})_6$ solution = 0.01 grm. $\text{K}_4\text{Fe}(\text{CN})_6 \cdot 3\text{H}_2\text{O}$ = 0.0037 grm. CN.

Two applications of this analytical method to the analysis of by-products from the destructive distillation of coal are discussed in the sections on the Determination of Cyanogen Compounds in Coal Gas and the Determination of Ferrocyanide in Spent Oxide.

Distillation with Cuprous Chloride.—This method, due to H. E. Williams, is exceedingly useful on account of the large number of ferrocyanogen compounds to which it is applicable. It is particularly useful for the analysis of the insoluble ferrocyanides. The analytical directions given by Williams (*Chemistry of Cyanogen Compounds*, 1915, p. 361) are as follows:

"The principle of the method rests on the fact that when cuprous chloride is added to an acid solution of a ferrocyanide, complex cuprous cyanides are formed which are easily decomposed by boiling with a dilute mineral acid, with evolution of hydrocyanic acid and regeneration of the cuprous chloride, which is thus available for the decomposition of a further quantity of ferrocyanide. As these two reactions can proceed simultaneously, the complete decomposition of the ferrocyanide may be accomplished in one operation and with a relatively small quantity of cuprous chloride."

"The best method of carrying out the process is to take 0.5 grm. of the ferrocyanide dissolved in water, or a volume of solution containing an equivalent quantity, and place in a distilling flask of about 350 to 400 c.c. capacity and dilute to about 150 c.c. Weigh out 0.1 to 0.05 grm. of cuprous chloride, wash in a small beaker with a little dilute sulphuric acid to remove any basic cupric chloride that is generally present, and dissolve in a few drops of hydrochloric acid, or in a saturated solution of sodium or potassium chloride, and add to the ferrocyanide solution in the distilling flask with gentle agitation. A white precipitate is produced of a double cuprous ferrocyanide; 50 c.c. of 4*N* sulphuric acid are then added, the flask connected to a condenser to which are attached two absorption flasks containing a dilute solution of sodium or potassium hydroxide

and gently distilled. After a few minutes' boiling the white precipitate changes to light blue, due to the precipitation of the double ferrous ferrocyanide; this precipitate is then slowly decomposed, and ultimately the solution becomes quite clear, the whole of the ferrocyanide being decomposed and the cyanogen driven off as hydrocyanic acid, leaving a solution containing ferrous sulphate and bisulphate of the alkali metal used, together with the excess of sulphuric acid and the regenerated cuprous chloride."

"The alkaline solution in the absorption flasks is carefully washed out into a titrating beaker and a few drops of a 10% solution of potassium iodide added, and the liquor titrated with *N*/10 silver nitrate solution, while being agitated, until the first appearance of a permanent opalescence."

In the case of the insoluble ferrocyanides, such as those of the heavy metals, a sample weighing 0.5 grm. is weighed into the distilling flask with 150 c.c. of water, and the requisite quantities of dilute sulphuric acid and cuprous chloride are added. The hydrocyanic acid is then distilled off in the usual way. Ferrocyanides intermixed with other bodies, such as Prussian blue in dyed or weighted silk or in spent oxide of iron, can also be estimated direct by this method.

Benzidine Method.—A recent method (*J. Chem. Soc.*, 1924, **125**, 240) is that of W. M. Cumming who employs a benzidine salt for the analysis of the soluble ferrocyanides, basing the determination upon the fact that the ferrocyanide of benzidine $3\text{C}_{12}\text{H}_{12}\text{N}_2 \cdot \text{H}_4\text{Fe}(\text{CN})_6\text{H}_2\text{O}$, is soluble in water only to the extent of 1 part in 12,000 at 17°. For gravimetric analysis the benzidine salt is added in excess to the ferrocyanide solution, the white precipitate is filtered off, washed with water, dried in an air bath, ignited, and the residue of ferric oxide weighed. The factors used for converting the found weight of Fe_2O_3 into weight of anhydrous original salts are 4.599, 3.799 and 3.649, respectively, for potassium, sodium and calcium ferrocyanides.

For volumetric analysis a standard solution of benzidine dihydrochloride is made by dissolving a known weight of the pure salt in water. This neutral solution is used for the titration of neutral ferrocyanide solutions. When somewhat less than the approximate volume of benzidine required for complete precipitation has been added, with constant stirring, a drop of the mixture is placed on filter paper. The white precipitate decomposes in the air, and a blue centre surrounded by a colourless ring of solution forms.

A drop of bromine indicator (prepared by dissolving a few drops of bromine in 100 c.c. of water and adding just sufficient sodium carbonate to decolorise the liquid) is now placed on the filter paper so that it can spread and touch the colourless ring. When the end-point is reached a yellow coloration will appear at the juncture of the two liquids. The tests must be made as quickly as possible, and the yellow coloration must be produced in the colourless ring.

Standard Method of Prussiate Analysis.—At a recent conference of the chemists of the European Prussiate Manufacturers a standard method of analysis was agreed upon, and it was arranged that this method should be published in the various countries of the different workers (see *Analyst*, 1929, 54, 38). At this conference the different methods of ferrocyanide estimation were critically examined, and the zinc sulphate method was finally accepted as the standard method for the settlement of disputes between manufacturer and customer. The findings and the method as agreed upon were as follows:

(1) It was generally agreed that the permanganate method could not be used, as the presence of oxidisable impurities would give false results.

(2) It was agreed by all that the method for the estimation of ferrocyanide by distillation as hydrocyanic acid was good. Doubts were raised against this method, however, as lower results were more frequently obtained by it than by other methods, and it did not seem practicable to accept it as a general standard method which was to be made available for public laboratories. The method in which mercuric chloride was used was preferred to that in which cuprous chloride was used.

(3) The copper sulphate method was generally condemned, as it had been found that the results were not reliable, variable compounds being readily formed.

(4) It was agreed that the copper sulphate method of converting ferrocyanides into ferricyanides was good, so long as no sulphur compounds were present. Sulphur compounds caused the precipitation of copper, so that under certain conditions the method was not trustworthy.

(5) The zinc sulphate method was agreed to by all as being the best method.

Procedure.—*Zinc Sulphate Solution.*—About $\frac{1}{5}N$ solution (28.7 grm. per litre) is standardised against Kahlbaum's potassium ferrocyanide and the factor thus determined.

Ferrocyanide Solution.—10 grm. of the ferrocyanide are dissolved in water; and the solution diluted to 500 c.c. Of this solution, 50 c.c. are taken for analysis, diluted with water; and acidified slightly with dilute sulphuric acid (about 10 c.c. of $N/10$ pure sulphuric acid free from iron). A 15 per cent. solution of pure iron alum is used as indicator; the titration is carried out at 15° – 20° ; and the paper used for the titration must be free from iron, and, in particular, must be as ash-free as possible. In making the titration the zinc sulphate solution is added to the acidified potassium ferrocyanide solution. The end-point is determined by placing 2–3 drops of the titrated solution on a filter paper by means of a glass rod and leaving the paper for a few minutes to let the drops spread well. Then one or more drops of the indicator solution are placed on the paper at a distance of $1-1\frac{1}{2}$ cm. The end of the titration is shown when no trace of the blue colour appears at the junction of the two liquids. Special care must be taken to see whether any blue colour appears about 2–3 minutes after the two liquids come into contact. It is best, when placing the drops on the paper, to make a dent in it with a glass rod, as by this means the precipitate is kept back more easily and not carried to the outside edge. It is necessary to do this, for a blue colour will always appear when the iron salt comes into contact with the precipitated zinc ferrocyanide.

If such a quantity of the liquid to be titrated is taken for the drop tests that it considerably influences the final result, the titration must be repeated, and the zinc sulphate solution allowed to flow with the liquid to be titrated until just before the termination, with a minimum of drop reactions, *i.e.*, with a minimum loss of solution. It may be stated here that a drop taken on a thin glass rod represents about 0.10 c.c. of the titration solution.

Moisture.—A determination of the moisture content should follow. For this purpose a weighed quantity of the sample is placed in a drying oven at a temperature of 125° , to drive off the moisture and water of crystallisation until constant weight is obtained. The moisture content is calculated by subtracting the difference between the original weight taken and the dry weight.

Sampling.—As a special method of sampling it is recommended that a sample should be taken from each cask, so that for each ton of goods a kilo. sample is taken. This sample is to be divided into four containers and sealed. The sampling is to be done by a sworn sampler from the Chamber of Commerce or a similar authority.

Impurities.—The report also gives an outline of the methods of determining the most important impurities likely to be met with in commercial ferrocyanide. Fifty grm. of the ferrocyanide are dissolved in 250–300 c.c. of water, the solution treated with a slight excess of pure recrystallised zinc acetate, diluted to a litre, shaken thoroughly and filtered.

Chloride.—Take 100 c.c. of the clear filtrate, add a few drops of potassium chromate solution, and titrate with $N/10$ or $N/100$ silver nitrate solution in the usual manner. The end-point is sharp and distinct.

Sulphate.—Acidify 100 c.c. of the clear filtrate with pure hydrochloric acid, boil, and then add an excess of boiling barium chloride solution, filter, ignite the precipitate and weigh as barium sulphate.

Formate.—Take 100 c.c. of the clear filtrate, add an excess of mercuric chloride solution together with a few drops of acetic acid, boil for 30 minutes, filter on a weighed filter paper, wash, dry and weigh.

Weight of $\text{HgCl} \times 0.178 \times 10 \times 2 = \text{KHC}\text{O}_2$ per cent.

Weight of $\text{HgCl} \times 0.144 \times 10 \times 2 = \text{NaHC}\text{O}_2$ per cent.

Estimation of Cyanogen Compounds in Coal Gas

For the determination of cyanogen in coal gas the Chemical Committee of the Technical Section of the American Gas Association recommends the following procedure. (*Gas Chemists' Handbook*, Second Ed., 1922, 329). Pass the gas to be tested through a train of four gas-washing bottles (Drehschmidt's form is very satisfactory) arranged in series. Into each of the first three bottles place 15 c.c. of a 20% solution of copperas (technical ferrous sulphate) and 15 c.c. of a 20% solution of caustic potash, shaking each bottle vigorously to avoid formation of lumps. Add enough water to make a seal of $1\frac{1}{2}$ inches. Into the fourth bottle place sufficient water to give the required seal. Pass the gas through the absorption train at a rate of $1\frac{1}{2}$ to $1\frac{3}{4}$ cubic feet per hour, measuring the total amount

of gas passed with a meter. Record the volume of gas passed and its average temperature at the meter.

Transfer the contents of the bottles to a casserole, add 50 c.c. of a 20% solution of caustic potash, and digest over a low flame at a temperature just below the boiling-point until a test for ammonia with turmeric paper is negative. Cool the contents of the casserole, transfer to a 500 c.c. flask, and dilute to the mark. Mix thoroughly and filter through a dry filter into a dry beaker. Acidify a 100 c.c. portion of this filtrate with dilute sulphuric acid (10%), adding 10 c.c. in excess, and titrate with standard zinc sulphate solution, using 3% ferric alum solution as indicator and Schleicher and Schüll's Drop Reaction Paper, No. 601, or Baker and Adamson's Grade A filter paper, for the spot test. The method of titration is described on page 545.

The standard zinc sulphate solutions used in the titration should be equivalent to approximately 0.08 *grain* $K_4Fe(CN)_6 \cdot 3H_2O$ per c.c. Such a solution is prepared by dissolving 5.5 gm. of zinc sulphate ($ZnSO_4 \cdot 7H_2O$), adding 5 c.c. of concentrated H_2SO_4 , and diluting to one litre.

The meter reading at the observed temperature is converted into volume at the standard temperature of 60° F. The cyanogen content of the gas is then obtained by the relation

$$\frac{5 \times \text{factor of Zinc Sulphate solution in grains of Cyanogen} \times 100}{\text{Corrected volume of gas passed}} = \text{grains CN in 100 cubic feet of gas.}$$

Estimation of Cyanogen Combined as Ferrocyanide in Spent Oxide

(Gas Chemists Handbook 1922, p. 324)

The various methods used for the sampling of coal may be applied to advantage in sampling spent oxide. A 5-pound sample shall be delivered to the laboratory. This sample is then reduced in size by the usual method of quartering.

Moisture.—"Dry 30 gm. of oxide for 9 hours in an oven previously heated to 50 or 60° C. Do not allow the temperature of the oven to exceed 60° C.

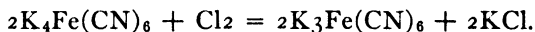
Procedure of Analysis.—"Grind the dried oxide so that it passes through an 80-mesh sieve. Weigh out 25 gm. of this into a 250 c.c. beaker. Add 100 c.c. of a 10% caustic potash solution and allow it

to digest, with frequent stirring, for 18 to 20 hours at room temperature. At the end of this time, transfer the entire contents of the beaker to a 500 c.c. flask and dilute to 510 c.c. (10 c.c. for the oxide). Filter through a dry filter. Take 100 c.c. of the filtrate and run it into a solution of ferric chloride—50 c.c. This ferric chloride solution consists of 60 grm. of ferric chloride and 100 c.c. of concentrated hydrochloric acid (sp. gr. 1.19) diluted to 1,000 c.c. with distilled water. After the blue settles a little, filter and wash the blue with boiling water, until it is free from thiocyanates. Then place the blue and filter paper in a beaker and add 25 c.c. of caustic potash (10% solution). Immediately after decomposition, transfer the mixture to a 250 c.c. flask and dilute to 250 c.c. Thoroughly mix, and filter through a dry filter into a dry beaker. Take 100 c.c. of this filtrate, acidify with 10% sulphuric acid (test with litmus), adding an excess of 10 c.c. of the acid, and 10 c.c. of a saturated solution of potassium sulphate. Titrate with standard zinc solution, the operation being the same as in the standardisation of the zinc solution."

The Ferricyanides

The ferricyanides are produced by the action of oxidising agents on the ferrocyanides. The potassium salt is the only salt of ferricyanogen of any considerable commercial importance although, in recent years, some attention has been given to the manufacture of the sodium salt.

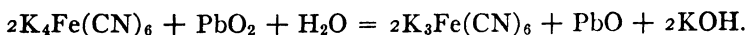
Potassium Ferricyanide.—*Manufacture.*—The oldest process for the oxidation of potassium ferrocyanide to potassium ferricyanide, and the process most commonly used, employs chlorine as the oxidising agent, the gas being allowed to act upon the dry salt or upon its solution in water. The reaction is represented by the equation



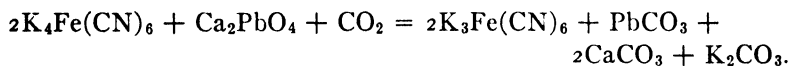
In the dry process the finely ground ferrocyanide is exposed to the action of chlorine until tests with ferric chloride show complete disappearance of ferrocyanide. If the stream of chlorine is not at once interrupted the oxidation will proceed too far, and the product is coloured green or blue. When a solution of ferrocyanide is employed, as is usually the case, the solution contains 10% sodium ferrocyanide, and the gas is added until oxidation is just complete.

To obtain a pure ferricyanide from the product of the chlorine oxidation it must be separated from the alkali chloride, which is one of the products of the reaction, and from the traces of ferro-blues produced by local over-oxidation. The separation is not easy, partly because ferricyanide solutions are somewhat unstable, making concentration by crystallisation undesirable, and also because of the fact that the alkali chloride is difficult to separate from the alkali ferricyanide.

To avoid the necessity of separation of the ferricyanide and chloride Schönbein recommended oxidation with lead peroxide. The reaction in this case is—



For each mol. of ferricyanide produced, one mol. of alkali hydroxide is formed, and since this would decompose the ferricyanide, it must be removed as formed. This is conveniently done by continuously conducting carbon dioxide into the mixture during the reaction. Kassner improved the process by substituting calcium plumbate for the lead peroxide. The reaction in this case is



To remove the potassium carbonate the calculated amount of calcium ferricyanide solution is added for the complete precipitation of carbonate, the calcium ferricyanide having been produced by oxidation of calcium ferrocyanide. This process yields potassium ferricyanide in substantially pure solution.

Potassium permanganate may be substituted for the lead compound, and a fairly pure ferricyanide solution may be secured when the ferrocyanide is supplied in a suitably proportioned mixture of the potassium and the calcium salts.

More recently, electrolytic oxidation has been substituted for the chemical oxidising agents. When a solution of potassium ferrocyanide is electrolysed in a diaphragm cell ferricyanide is formed at the anode. Caustic potash is simultaneously produced and may be removed by use of carbon dioxide, as described in earlier paragraphs. The electrolytic process has the important advantage over the chemical oxidations that the ferricyanide may be recovered in solid form from the anode compartment by addition of solid potassium ferro-

cyanide until the solution becomes saturated with the ferri-compound and precipitation of the solid ferri-salt occurs.

Properties and Uses.—Potassium ferricyanide or “red prussiate of potash” may be obtained from aqueous solution in the form of deep red prisms. It crystallises without water.

The solubility of the salt at various temperatures is as follows:

Temperature, ° C.	Grm. salt in 100 grm. water
4.4	33.0
10	36.0
15.6	40.8
37.8	58.8
100	77.5
104.4	82.6

Solutions of potassium ferricyanide are only moderately stable; in alkaline solutions it is a powerful oxidising agent.

Potassium ferricyanide is used in the printing and dyeing of textiles and in the manufacture of blue-print paper. In the textile industry the ferricyanide is used primarily as an oxidising agent in aniline blacks and for certain oxidation discharges. When printed on light indigo-dyed cloth it discharges the blue when passed through a hot solution of caustic soda. In the manufacture of blue-print paper which will print white lines on a blue background the paper is sensitised with a solution containing potassium ferricyanide and ferrous ammonium citrate. As an example, two solutions are prepared, one containing 20 parts of ferrous ammonium citrate in 100 parts of water and another containing 16 parts of potassium ferricyanide in 100 parts of water; these are mixed immediately before using, and the mixed solution applied to the paper.

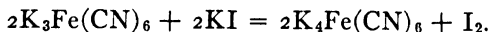
Detection and Estimation of Ferricyanides

Detection.—Neither the iron nor the cyanogen of ferricyanides can be recognised by the ordinary tests. The soluble ferricyanides are red in colour and yield a yellow to orange solution. They are moderately soluble in alcohol, differing in this respect from the ferrocyanides, which are precipitated from solution by addition of alcohol. With ferric iron, ferricyanide yields no precipitate; ferrous iron yields a deep blue precipitate which is insoluble in acids. Certain of the metallic ferricyanides exhibit characteristic colours which differ from those of the ferrocyanides.

Metal	Colour of the precipitate yielded by	
	Ferrocyanide	Ferricyanide
Bismuth	White	Light brown
Cadmium	White	Yellow
Cobalt	Green to gray	Brown red
Copper	Red-brown	Yellow-green
Mercury(ous)	White	Red-brown turning white
Mercury(ic)	White
Nickel	Greenish-white	Yellowish-green
Silver	White	Red-brown
Zinc	White	Orange

Ferricyanides are readily reduced to ferrocyanides by most reducing agents. When boiled with yellow mercuric oxide and water, ferricyanides are completely decomposed, with formation of mercuric cyanide and precipitation of oxide of iron. When treated with a nitrite and acetic acid ferricyanides are converted into nitroprussides.

Estimation.—(a) *Iodometric Method.*—In hydrochloric acid solution potassium ferricyanide is reduced by potassium iodide, liberating free iodine which may be determined by titration with thiosulphate

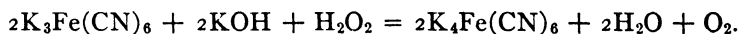


The reaction is reversible, but may be made complete by addition of a slight excess of zinc sulphate solution to precipitate the ferrocyanide. Williams (*loc. cit.*) specifies the following conditions for the analysis: A solution of the ferricyanide containing about 0.5 gm. is diluted with distilled water to 500 to 700 c.c., then 10 to 15 c.c. of concentrated hydrochloric acid (sp. gr. 1.19), a solution of 1 to 2 gm. of potassium iodide and 10 c.c. of a strong solution of zinc sulphate are added. It is an advantage to add also 10 to 15 c.c. of a saturated solution of potassium chloride to cause the precipitated zinc ferrocyanide to settle more readily and prevent it becoming too gelatinous. The mixture, after being well shaken and allowed to stand three or four minutes, is titrated with standard *N*/10 sodium thiosulphate solution until nearly all the free iodine is removed, and, after standing for another two or three minutes, the titration is completed.

If both ferro- and ferricyanide exist in the same solution, the ferricyanide may be estimated by the method described above, and the ferrocyanide by taking a fresh portion of the solution, diluting it to 600 to 700 c.c., adding 10 to 15 c.c. of concentrated hydrochloric acid and 40 to 60 c.c. of strong sodium acetate solution, followed by $N/10$ permanganate in slight excess. The excess of permanganate is then determined by first adding potassium iodide solution and then titrating the liberated iodine with $N/10$ sodium thiosulphate.

(b) *Permanganate Method*.—The ferricyanide may be completely reduced to ferrocyanide, and the latter determined by titration with permanganate, as described on page 543. Four grm. of the salt are dissolved in about 200 c.c. of water in a 500 c.c. graduated flask. Caustic potash is now added until the mixture is strongly alkaline; it is then heated to boiling and a concentrated solution of ferrous sulphate added. A brown precipitate of ferric hydroxide is formed which later turns black. When this colour change has occurred the mixture is cooled, made up to the mark, and filtered through a dry paper, and 100 c.c. of the filtrate are titrated with permanganate after acidification, as described for the permanganate titration of ferrocyanide.

(c) *Hydrogen Peroxide Method*.—Quincke (*Z. anal. Chem.*, 1892, 31, 1) has shown that the action of alkali hydroxide and hydrogen peroxide on potassium ferricyanide proceeds quantitatively according to the equation:



The volume of oxygen evolved is therefore a measure of the ferricyanide present.

The best method of operating is to introduce 5 to 10 c.c. of the solution of the ferricyanide into the closed tube of a nitrometer filled with mercury, and then to run in an equal amount of strong solution of sodium hydroxide through the tap. This is followed by a solution of hydrogen peroxide, and the contents of the nitrometer are mixed by agitation; more hydrogen peroxide is then added to insure completion of the reaction, and water equal to the combined aqueous liquids is poured into the open limb, the level of the mercury in the two limbs adjusted, and the volume of gas read off. 1 c.c. of oxygen at 0° and 760 mm. = 0.02945 grm. of potassium ferricyanide.

The Nitrosoferricyanides

By substitution of a nitroso group for one of the cyanogens of the ferricyanides, a nitrosoferricyanide or nitroprusside is obtained. Nitroprussic acid, $\text{H}_2\text{Fe}(\text{CN})_5\text{NO}$, is formed by treating potassium ferro- or ferricyanide with nitric acid or by the addition of sulphuric acid to a soluble nitroprusside. It forms red-brown leaflets which are very soluble in water, but yield an unstable solution. The acid has been shown by conductivity measurements to be somewhat stronger than sulphuric acid (Burrows and Turner, *J. Chem. Soc.*, 1921, 119, 1450).

The sodium salt of the acid is usually prepared, on account of the ease with which it may be produced in pure form. Williams recommends the following procedure: 48.5 grm. of ground crystallised sodium ferrocyanide are placed in a 1,000 c.c. flask, 250 c.c. of nitric acid (1:1) are added, and the reaction allowed to proceed in the cold. When the first action is over the mixture is heated to 100° on a water bath and is kept at this temperature until a drop of the liquid produces neither a blue nor a green coloration with a solution containing a mixture of ferrous and ferric salts. The liquid is diluted conveniently, neutralised with precipitated calcium carbonate, filtered, and the solution evaporated and set aside for crystallisation. Large crystals of sodium nitroprusside are obtained which may require purification by recrystallisation. The pure salt, $\text{Na}_2\text{Fe}(\text{CN})_5\text{NO}, 2\text{H}_2\text{O}$, crystallises in ruby red monoclinic prisms which dissolve in 2.5 parts of water at 16° .

The alkali nitroprussides are fairly stable in aqueous solution; the alkaline earth salts are somewhat less stable. Soluble nitroprussides are unchanged by ferric salts. With silver and certain other heavy metal salts they yield flesh-coloured precipitates.

The chief interest attaching to the nitroprussides is the beautiful, but transient, violet colour which they yield with soluble sulphides. This reaction forms a delicate test both for sulphides and for nitroprussides.

Sodium nitroprusside is also used as a test for creatinine, which in presence of alkali hydroxide gives a ruby-red coloration, changing in a few minutes to a straw colour. Acetone also gives a red colour with alkaline nitroprusside solution, and other ketones and aldehydes give reactions ranging from yellowish-red to violet.

The Carbonyl-ferrocyanides

One of the cyanogen groups in the ferrocyanogen ion can be replaced by carbonyl, forming salts of carbonyl ferrocyanide, *e. g.*, $\text{K}_3\text{Fe}(\text{CN})_5\text{CO}$. The carbonyl ferrocyanides may be produced by heating a solution of potassium ferrocyanide to 135° in an atmosphere of carbon monoxide for two or three days, or by the action of strong sulphuric acid on ferrocyanides. They are more easily obtained, however, from the cyanogen products recovered from coal gas, in which they may be found in considerable amounts. For their recovery from the crude mother liquors of ferrocyanide manufacture Williams recommends the following procedure: After evaporation of the solutions and separation of the greater part of the ferrocyanide by crystallisation the residual mother liquor is acidified with hydrochloric or dilute sulphuric acid and the solution is precipitated by addition of a ferric salt. The precipitate is a mixture of the iron salts of ferrocyanide and carbonyl-ferrocyanide and is separated from the solution by filtration and thorough washing. The ferrocyanide and the carbonyl-ferrocyanide are separated by converting them into calcium salts by boiling with milk of lime, and the ferrocyanide is precipitated by addition of sufficient ammonium carbonate to form calcium ammonium ferrocyanide. Calcium carbonyl-ferrocyanide is now obtained from the resulting purified solution by evaporation and crystallisation.

The alkali and alkaline earth carbonyl-ferrocyanides are light yellow in colour, and their solutions are stable. Most of the metallic carbonyl-ferrocyanides are insoluble. The cobalt salt is obtained as a reddish-pink precipitate; the cupric salt is light green; the silver salt and the zinc salts are both white. The lead salt is soluble, and advantage is taken of this property in the estimation of the carbonyl-ferrocyanides.

The precipitate obtained by the addition of a ferric salt to a soluble carbonyl-ferrocyanide is the most characteristic reaction of the carbonyl-ferrocyanides. The colour of this compound, which is a characteristic violet with a high bronze lustre, readily distinguishes it from the ferrocyanides. Like the ferrocyanides, the carbonyl-ferrocyanides when precipitated with ferric salts carry with them in combination small amounts of the alkali metals.

The Detection and Estimation of Carbonylferrocyanide.—For the analytical separation of carbonyl-ferrocyanide from the ferro-

cyanide with which it is usually associated advantage is taken of the fact that the lead salt of the former is soluble in neutral or acid solutions. Williams proceeds as follows:

To the neutral or slightly acid solution lead nitrate or acetate is added in excess. By this means the ferrocyanide is precipitated as the insoluble lead salt, while the carbonyl-ferrocyanide remains in solution. The precipitate is filtered off and thoroughly washed, the excess lead removed from the filtrate by the addition of a solution of sodium or potassium sulphate, and the precipitated lead sulphate filtered off and washed. The carbonyl-ferrocyanide in the filtrate and washings is then precipitated by the addition of an excess of a ferric salt solution, the violet ferric carbonyl-ferrocyanide filtered off on a large filter and thoroughly washed. The precipitate, together with the paper, is then transferred to the beaker in which it was precipitated, and decomposed with 100 c.c. of a 3% solution of potassium hydroxide, the mixture being well stirred with a glass rod. It is then again filtered and washed, and the filtrate estimated by titration with copper sulfate, the procedure recommended on page 544 for the titration of ferrocyanide being followed exactly. The copper sulfate solution must, of course, be standardised against a carbonyl-ferrocyanide, and for this the calcium potassium carbonyl-ferrocyanide is recommended. The crystalline salt is $\text{CaKFe(CN)}_5\text{CO}_5\text{H}_2\text{O}$. For standardisation a weighed quantity of the pure salt is boiled with potassium carbonate, filtered, and the filtrate titrated with the copper salt.

The Cobalticyanides

This very permanent class of double cyanides is chiefly of interest from its application to the separation of nickel and cobalt. When an alkali cyanide is added to a solution of cobalt, brownish white cobaltous cyanide Co(CN)_2 is formed. With excess of alkali cyanide, red alkali cobaltocyanide, $\text{K}_4\text{Co(CN)}_6$, is formed. On heating this solution, it is quickly converted, with evolution of hydrogen, into the colourless and very stable alkali cobalticyanide, $\text{K}_3\text{Co(CN)}_6$, analogous to the ferricyanide. A more complete reaction occurs on treating the cold solution with chlorine or bromine.

The resulting solution of alkali cobalticyanide is not decomposed by boiling with mercuric oxide. This reaction enables nickel to be separated from cobalt; the former is wholly precipitated and remains

as NiO on igniting the precipitate. The same reaction distinguishes the cobaltcyanides from the ferro- and ferri-cyanides.

The cobaltcyanides are compounds of great stability. They may be boiled with strong hydrochloric or nitric acids without decomposition, and are only very slowly attacked by strong sulphuric acid. Their aqueous solutions give no precipitates with ferric salts, nor with salts of lead or mercuric mercury. Mercurous salts completely precipitate cobaltcyanides from neutral solutions, the precipitate leaving Co_3O_4 on ignition.

The Platinocyanides

Platinous cyanide, $\text{Pt}(\text{CN})_2$, is one of the most stable of the metallic cyanides, so stable, in fact, that it may be produced by precipitation of solutions of mercuric cyanide with platinous chloride. When dissolved in an excess of alkali cyanide the platinocyanides are produced, these with the iron cyanogen compounds and the cobaltcyanides constituting the three important members of the class of stable complex cyanides.

The alkali and alkaline earth platinocyanides are of interest from their fluorescent properties. These properties are exhibited only by the solid salts and not by their solutions. They exhibit remarkable colours under the influence of various forms of radiation; the behaviour of barium platinocyanide towards the X-rays being particularly noteworthy.

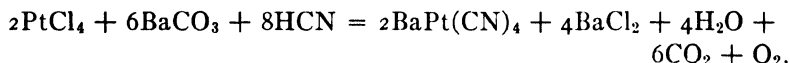
The platinocyanides of the light metals are soluble in water and crystallise well. Those of the heavy metals are mostly insoluble and can be prepared by precipitation. The platinocyanides are extremely stable, even sulphuric acid decomposing them but slowly. When treated in solution with nitric acid, chlorine or bromine, the platinocyanides form addition products. Thus on passing chlorine into a hot solution of potassium platinocyanide, the compound $\text{K}_2\text{Pt}(\text{CN})_4\text{Cl}_2 + 2\text{H}_2\text{O}$ is deposited in colourless crystals on evaporation.

The platinocyanides are not decomposed by digestion with mercuric oxide. Mercuric chloride throws down white mercuric platinocyanide, $\text{HgPt}(\text{CN})_4$. Mercurous platinocyanide is blue, but changes to white on boiling, the mercurous salt being converted into a mercuric salt. Copper platinocyanide is green; lead platinocyanide is yellow; silver platinocyanide is white and curdy.

Potassium Platinocyanide.— $\text{K}_2\text{Pt}(\text{CN})_4 \cdot 3\text{H}_2\text{O}$ is obtained by evaporating to crystallisation a solution of platinous chloride in potassium cyanide. It crystallises in rhombic crystals which are yellow by transmitted light and blue by reflected light; the crystals effloresce in dry air and finally become white. The salt is readily soluble in hot water but is in great part deposited on cooling. Cold concentrated sulphuric acid decomposes it slowly.

Potassium platinocyanide forms a number of easily crystallisable double salts with other soluble platinocyanides. The double potassium sodium salt crystallises in monoclinic crystals, deep orange by transmitted light and purple by reflected light. The potassium-strontium and potassium-lithium salts are also of interest on account of their colours.

Barium Platinocyanide.— $\text{BaPt}(\text{CN})_4 \cdot 4\text{H}_2\text{O}$ may be prepared by passing hydrocyanic acid vapours into a solution of platinic chloride holding barium carbonate in suspension, until oxygen and carbon dioxide cease to be evolved.



Barium platinocyanide forms monoclinic prisms which appear green in the direction of the principal axis, but sulphur yellow with a blue-violet sheen in the direction at right angles to the axis. The salt dissolves in about 33 parts of cold water, but is considerably more soluble at the boiling point.

Magnesium Platinocyanide.— $\text{MgPt}(\text{CN})_4 \cdot 7\text{H}_2\text{O}$ is obtained by precipitating the barium salt with magnesium sulphate. It crystallises in large prisms which are red by transmitted light and green by reflected light. When crystallised from alcohol and from water at 70° a yellow salt containing six molecules of water is obtained, which at 100° is converted into a white hydrate containing $2\text{H}_2\text{O}$, and this at 180° gives a yellow anhydrous salt.

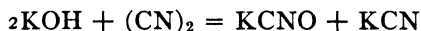
Owing to their strongly developed crystalline characteristics the platinocyanides are occasionally employed for the isolation of certain organic bases. Levy (*Proc. Camb. Phil. Soc.*, 1907, **14**, 159) has described the properties of the platinocyanides of guanidine, $(\text{CN}_3\text{H}_5)_2\text{H}_2\text{Pt}(\text{CN})_4$, and of nitron $(\text{C}_2\text{OH}_{16}\text{N}_4)_2\text{Pt}(\text{CN})_4$.

THE OXYCYANOGEN COMPOUNDS

CYANIC ACID AND THE CYANATES

The metallic cyanates are obtainable by the following reactions:

1. By passing cyanogen gas into the solution of the hydroxide of an alkali or alkali earth metal:—



2. By the electrolysis of a solution of the corresponding cyanide, the cyanate being formed at the anode.

3. By fusing a cyanide or ferrocyanide with an oxidising agent, such as manganese dioxide, red lead, litharge, or potassium dichromate.

4. By the action of a highly alkaline solution of a hypochlorite on urea.

5. By the action of a hypobromite or an alkaline solution of permanganate on a cyanide.

Cyanic Acid.—The free acid, HCNO, cannot be prepared by the action of mineral acids on metallic cyanates, since, at the moment of formation, it takes up water, with formation of carbon dioxide and ammonia. It may, however, be prepared by passing dry hydrochloric acid gas over silver cyanate. It is also prepared by heating its polymer, anhydrous cyanuric acid, or by heating a mixture of urea with phosphoric anhydride.

Cyanic acid is a colourless liquid having an extremely pungent odor resembling that of sulphurous or glacial acetic acid. Its vapours are strongly lachrymatory and the acid itself will cause severe burns to the skin. It is extremely unstable, becoming rapidly polymerised into a snow-white insoluble substance called cyamelide. Aqueous solutions of the acid are only stable at very low temperatures; at higher temperatures they decompose into carbon dioxide and ammonia.

Two isomeric forms of cyanic acid are capable of existence,

represented by the following formulae:— $\begin{array}{c} \text{C} \equiv \text{N} \\ \diagdown \\ \text{OH} \end{array}$ the hydroxyl

$\begin{array}{c} \text{C} = \text{O} \\ \diagdown \\ \text{NH} \end{array}$ derivative of cyanogen, and $\begin{array}{c} \text{C} \equiv \text{N} \\ \diagup \\ \text{OH} \end{array}$ carbylimide. These are known, respectively, as cyanic and isocyanic acids.

The constitution of cyanic acid has been a subject of much discussion. Warner and Fearon (*J. Chem. Soc.*, 1920, 117, 1356) assume that the acid is an equilibrium mixture, as represented by the

by rise in temperature
 relation $\text{HOCN} \rightleftharpoons \text{HNCO}$. Of these tautomeric
 by fall in temperature

forms, the keto form, HNCO , polymerises to cyanuric acid, and the enol form, HOCN , to cyamelide. Experiments made on the polymerisation of liquid cyanic acid at various temperatures showed that with increase of temperature the proportion of cyanuric acid increased regularly and that of cyamelide decreased, indicating that with increase in temperature the equilibrium of the keto and enol forms of cyanic acid shifted towards the keto form. At 0° cyanic acid is calculated by these experiments to be 60% of the enol and 40% of the keto modification.

Difference of opinion also exists concerning the constitution of the metallic cyanates. The alkyl cyanates are almost unknown, as they polymerise with great facility into the corresponding cyanurates. The alkyl isocyanates are obtainable as volatile pungent liquids readily polymerising to isocyanurates.

Ammonium Cyanate.—Ammonium cyanate may be obtained by decomposing silver cyanate with a solution of ammonium chloride, or barium, lead or potassium cyanate with ammonium sulphate. Ammonium cyanate is extremely unstable, being converted into urea by boiling or evaporating the solution. This transformation, first noted by Wöhler, is a classic instance familiar to all students of the history of chemistry.

Potassium Cyanate.—Potassium cyanate, KCNO , is the most important salt of cyanic acid. It is formed by passing cyanogen or cyanogen chloride into potassium hydroxide, by melting potassium ferrocyanide with potassium dichromate, or by the oxidation of a cold solution of potassium cyanide with potassium permanganate.

It crystallises in colourless scales fusible without decomposition. It is readily soluble in water and moderately soluble in boiling 95% alcohol, but not in absolute alcohol. On evaporation of its aqueous solution it suffers hydrolysis according to the following equation:—



On addition of moderately concentrated sulphuric or hydrochloric acid to potassium cyanate the greater part of the cyanic acid liberated

is decomposed into ammonia and carbon dioxide. Traces of the acid may escape this change, and hence the carbon dioxide evolved has an exceedingly pungent odour resembling that of sulphurous acid and most powerfully affects the eyes.

Of the heavy metal cyanates, silver cyanate is white and insoluble in water, but easily soluble in dilute nitric acid. Cobalt salts precipitate with potassium cyanate in dark blue crystals, $\text{Co}(\text{CNO})_2 \cdot 2\text{KCNO}$, a reaction employed in the qualitative analysis of cyanate mixtures. With copper a green precipitate, and with lead a white precipitate is produced.

Detection and Estimation of Cyanates

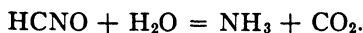
Detection.—As already stated, sulphuric acid, both concentrated and dilute, decomposes cyanates, forming ammonia and evolving carbon dioxide.

Silver nitrate forms a white cheese-like precipitate which is soluble in ammonia and nitric acid, thus differing from silver cyanide.

Cobalt acetate solution is coloured deep blue by the addition of cyanate solutions. The blue compound is hydrolysed when the solutions are diluted, and the colour disappears; it is, however, stable in alcohol. Nearly all commercial cyanide contains cyanate, but the hydrocyanic acid must be driven off before the presence of cyanates can be detected. According to Schneider (*Ber.*, 1895, **28**, 1540) the test should be made by dissolving about 3 to 5 gm. of the commercial cyanide in about 30 to 50 c.c. of cold water and passing carbon dioxide through the solution for 1.5 hours to drive off the hydrocyanic acid; 1 c.c. of the liquid is treated with 25 c.c. of absolute alcohol, which precipitates the carbonate formed, and the liquid is then filtered. A few drops of acetic acid are added to the filtrate, and then a few drops of an alcoholic solution of cobalt acetate. If 0.5% cyanate is present in the commercial cyanide, it can be plainly detected.

Fosse (*Compt. rend.*, 1920, **171**, 635) describes a test which depends upon the conversion of ammonium cyanate into urea. To test for cyanate in a solution take two samples, heat one of them for one hour with ammonium chloride; to 2 c.c. of each add 4 c.c. of acetic acid and 0.3 c.c. of 10% methylxanthidrol; if the weight of xanthylurea is greater in the second sample than in the first, the unknown contains cyanate.

Estimation.—Cyanates are usually estimated by hydrolysing them in acid solution and determining the ammonia produced—



About 0.5 grm. of the material to be analysed is weighed into a Kjeldahl flask and dissolved in 100 c.c. of water. 25 c.c. of 10% sulphuric acid are now added, and the mixture is boiled gently until its volume has been reduced about one half. The mixture is then cooled, sufficient caustic soda is added to make it strongly alkaline, the flask is set into the ammonia distillation rack, and the ammonia is boiled off into standard acid.

In cases where this method cannot be applied directly on account of presence of other compounds capable of liberating ammonia under the given conditions, the cyanate may be separated from the solution by precipitation of silver cyanate. The precipitate, which will contain not only the cyanate but also any cyanides, ferrocyanides or chlorides present, is filtered off and washed. It is then digested for one hour on the water bath with dilute nitric acid, filtered, and the cyanate originally present in the precipitate determined either by titrating the dissolved silver by the Volhard titration or by distilling off the ammonia resulting from the decomposition of the cyanate.

CYANURIC ACID AND THE CYANURATES

Cyanuric acid is a tribasic acid whose constitution is represented by the formula $(\text{CN})_3(\text{OH})_3$. It is the trimer of ordinary cyanic acid and may be produced by the polymerisation of the latter in absence of water, by dry distillation of urea, or by the action of chlorine upon urea.

According to R. von Walther (*J. prakt. Chem.*, 1909, **79**, 1268) cyanuric acid may be produced by heating urea at 220° with twice its weight of zinc chloride. The mass is cooled and decomposed with hydrochloric acid, and the crystals which separate are purified by recrystallisation from hot water. Béhal (*Bull. Soc. Chim.*, 1914, **15**, 149) passes a rapid stream of chlorine into 600 grm. of urea melted at $110\text{--}150^\circ$ and heats the solid product at $190\text{--}240^\circ$ for about 36 hours until no further alkaline gas is evolved. He then treats the solid in 5 litres of boiling water with 200 c.c. of concentrated ammonia, followed by gradual addition of 20% ammoniacal

copper sulphate until the solution is coloured blue. Copper cyanurate is formed, which, when decomposed with nitric acid, gives the free acid in yield varying from 46 to 68% of the theory.

Cyanuric acid crystallises with two molecules of water which are lost when it is dried in air.

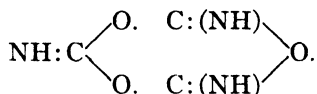
Detection of Cyanuric Acid.—(a) By addition of ammoniacal cupric sulphate to a solution of cyanuric acid the amethyst-coloured copper ammonium cyanurate is produced.

(b) When an aqueous solution of the acid is warmed with concentrated sodium hydroxide on a watch glass, fine needles of the salt $\text{Na}_3\text{C}_3\text{N}_3\text{O}_3$ are formed. These disappear on cooling unless the solution is too concentrated (Hofmann, *Ber.* 1870, 3, 770).

(c) When the acid is heated dry the characteristic odour of cyanic acid is observed.

CYAMELIDE

Cyamelide, like cyanuric acid, is formed by polymerisation of cyanic acid. When heated it is converted back to cyanic acid. It is a colourless compound, insoluble in water, in weak acids and in alcohol, but soluble in alkalis. Its structure, according to Hantzsch (*Ber.*, 1905, 38, 1016), is represented by the formula—



As stated earlier, Werner believes that cyamelide is the polymer of the enol form of cyanic acid, HOCN , differing from cyanuric acid, which is the polymer of the keto modification.

FULMINIC ACID AND THE FULMINATES

When mercury is dissolved in an excess of nitric acid and the solution so obtained is added to ethyl alcohol, a reaction takes place in which the mercury salt of fulminic acid is precipitated.

The acid $\text{H}_2\text{C}_2\text{N}_2\text{O}_2$ is not known in the free state, and its constitutional formula is still a vexed question.

Scholl (*Ber.*, 1890, 23, 3505) considers that the properties and modes of formation of the fulminates are best expressed on the assumption that fulminic acid has the constitution of dioxyiminoethylene, OH.N:C:C:N.OH . Mercuric fulminate, according to Nef, is represented by the formula $(\text{C:NO})_2\text{Hg}$. According to Wieland

(*Ber.*, 1907, 40, 418), the production of fulminic acid from alcohol and nitric acid takes place in the following steps:—



Mercuric Fulminate.—The commercially important salts of fulminic acid are the mercury and the silver salts. The former is one of the most important chemical compounds in the explosive industry, being the active ingredient in nearly all exploders or detonators. According to Weaver (*Military Explosives*, 1917, p. 170) the manufacture of fulminate of mercury is conducted as follows:

Mercury and nitric acid (sp. gr. 1.38) are mixed in a glass carboy in equal parts by weight. The mercury dissolves in the nitric acid and, when completely dissolved, the contents are allowed to cool and the solution is emptied into a second carboy which contains 10 parts of ethyl alcohol.

The second carboy is kept at a temperature above 15° C. and is connected with a series of receivers which stand in a trough through which water circulates. The pipe from the last receiver leads into a condensing tower.

After a few minutes the reaction begins, the liquid boils and white vapours containing nitric and acetic esters, aldehyde, carbonic acid and hydrocyanic acid pass off. As the reaction proceeds the colour of the vapours changes from white to the red fumes of nitrogen tetroxide.

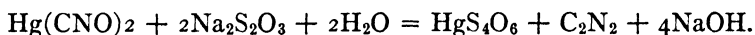
In about 15 minutes the crystals of fulminate of mercury separate from the solution in the form of small gray-colored needles. As soon as the reaction is completed the contents are allowed to cool, and are then poured out on a cloth filter stretched on a wooden form. The crystals are washed with water until the washings are neutral. The filter is then placed in a drying atmosphere until the mass of fulminate contains only 10 to 15% of water. The yield is about 125 parts of fulminate of mercury per 100 parts of metallic mercury.

Pure crystals of fulminate of mercury have a yellowish-white shade. The gray colour of the commercial product is due to small particles of unconverted mercury. Mercury fulminate is exceedingly sensitive to shock, particularly when dry. A moderate blow of

a hammer causes it to explode with a bright flash and gray fumes of mercury. The volume of gases evolved is 1,340 times the volume of the fulminate when calculated to ordinary temperatures and pressures. Larger crystals of mercury fulminate are more sensitive to shock than small crystals.

The great value of mercury fulminate as an exploder is due to the enormous pressure developed at its decomposition and to its suddenness. It is calculated that mercury fulminate exploding in its own volume gives a pressure of 28,750 kgrm. as compared with 12,376 kgrm. for nitroglycerin and 9,825 kgrm. for gun cotton.

Analysis of Mercuric Fulminate.—The analysis of mercury fulminate is based upon the reaction first described by Brownsdon, in which the fulminate is treated with a solution of sodium thiosulphate, whereby alkali is liberated which can be determined by titration. The equation is:—

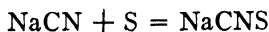


If the alkaline solution is allowed to stand for some time, the alkalinity of the solution gradually diminishes; this change is restrained by use of a large excess of thiosulphate or by potassium iodide. The double titration method of Philip, as described by Marshall (*Explosives*, Vol. II, p. 701), is as follows:—

50 c.c. of *N*/10 sodium thiosulphate solution are pipetted into a beaker and about 3 grm. of potassium iodide added to it; 0.3 grm. of fulminate is rapidly washed in, and the liquid is agitated until it is dissolved. The fulminate may be moistened beforehand with alcohol to prevent particles floating on the surface or the formation of lumps. A few drops of methyl orange solution are now added, and the solution is titrated with *N*/10 H_2SO_4 ; then a little starch solution is added, and it is titrated with *N*/10 iodine solution. 1 c.c. of *N*/10 thiosulphate is equivalent to 0.01423 grm. of mercury fulminate, and 1 c.c. of *N*/10 acid to 0.007115 grm. As a rule, the fulminate shows the same percentage in both titrations, but in abnormal samples the acidimetric titration sometimes gives a higher result. A good gray fulminate will show 98.0–99.5% of the pure substance; white fulminate generally gives results slightly lower, and fulminate purified with ammonia and acetic acid shows 100%, within the limits of experimental error.

THIOCYANIC ACID AND THE THIOCYANATES

- The thiocyanates are the sulphur analogues of the cyanates. They are most conveniently prepared by heating a solution of a cyanide with a polysulphide or with sulphur, addition of the sulphur taking place according to the equation



Like the cyanates, the thiocyanates exist in two isomeric forms, of which both the metallic and the alkyl salts are known. To ordinary thiocyanic acid the formula $\text{N}\equiv\text{C}-\text{S}-\text{H}$ is assigned. Thiocyanates of alkyl radicals are known, but they change with great facility into the isomeric isothiocyanates, to the typical acid of which the formula $\text{S}=\text{C}=\text{N}-\text{H}$ is assigned. Allyl isothiocyanate, the volatile oil of mustard, is a familiar example of the latter series.

According to Johnson (*J. Amer. Chem. Soc.*, 1906, **28**, 1454) the thiocyanates and isothiocyanates can be sharply distinguished by their behaviour towards thiol acids. Thiocyanates combine with thiolbenzoic and thioacetic acids, to form dithiourethanes, $\text{R}.\text{CONHCS}=\text{S}=\text{R}'$, or thiol esters, $\text{R}.\text{COS}=\text{R}'$, whilst isothiocyanates react to form substituted acid amides.

Notable quantities of thiocyanates occur in the saliva, pancreatic juice, bile and urine. Thiocyanates in the food are readily absorbed and remain as such in the body for a considerable time. The concentration of thiocyanates in the blood serum is less than that in the saliva. According to De Souza (*J. Physiol.*, 1907, **35**, 332) these substances are not a specific secretion of the salivary glands, but are merely a waste product in the blood, which is turned out by the salivary and other glands, together with and in a proportional concentration to the other salts in the secretions.

The principal commercial source of thiocyanates is the ammoniacal liquor and the spent oxide obtained in the purification of coal gas. In the process of the British Cyanides Company the whole of the cyanogen content of the coal gas is recovered as thiocyanate by passing the raw gas through spent purifier oxide over which a small amount of water is made to trickle. A strong solution of ammonium thiocyanate is recovered.

Most of the thiocyanates, including those of the alkalies, the alkaline earths, and most of the heavy metals, are soluble in water.

The salts of lead, silver and cuprous copper are very insoluble, whilst those of mercury and cupric copper are only sparingly soluble. The most familiar reaction of the thiocyanates is the formation of the intensely coloured compound with ferric iron, a reaction extensively used for the detection of both ferric iron and thiocyanate. The coloured compound so formed is more soluble in ether than in water and may be removed from aqueous solution by shaking with the latter solvent. Molybdenum salts also yield an intensely coloured compound with thiocyanate.

Although the addition of sulphur by the cyanide radical takes place with great ease, the reverse reaction is more difficult. Strong oxidising agents, such as nitric acid, split off the sulphur by oxidising it to sulphate, with liberation of free hydrocyanic acid. The sulphur may also be removed by fusing the dry thiocyanate with finely divided iron, a reaction employed on a commercial scale. Strong sulphuric acid completely decomposes the thiocyanates, yielding sulphates of ammonia and of the base.

Thiocyanic Acid.—According to Rück and Steinmetz (*Z. anorg. Chem.*, 1912, **77**, 51) pure gaseous thiocyanic acid may be prepared from potassium or barium thiocyanates by heating with potassium bisulphate, the gas being removed from the solids by a current of dry hydrogen under a vacuum of 40 mm. Pure HCNS is a colourless gas with an intense pungent odour. Compared with hydrocyanic acid, its toxicity is slight. Concentrated sulphuric acid decomposes it, forming a yellow mass. Dry acids or their anhydrides (oxalic acid, P_2O_5) cause no apparent change within some hours; aqueous or moist substances, even neutral salts, in contact with the gas cause slow decomposition. According to Rosenheim and Levy (*Ber.*, 1907, **40**, 2166) the melting point of the solid acid is about 5° . The liquid acid is yellow, but in a few minutes it becomes deep red and suddenly solidifies to a mass of slender yellow needles, heat being evolved. At 0° it is soluble in water in all proportions, practically without decomposition. The acid HCNS, itself, is mono-molecular, but the decomposition products have a much higher molecular weight. The nature of these cannot be definitely stated, although a silver salt corresponding to dithiocyanic acid has been obtained. Isodithiocyanic acid and isoperthiocyanic acid ($H_2C_2N_2S_3$) are described by Stokes and Cain (*J. Amer. Chem. Soc.*, 1907, **29**, 443).

Aluminium Thiocyanate.—Aluminium thiocyanate is obtained by the double decomposition of aluminium sulphate with barium thiocyanate. The solutions so obtained are uncrystallisable. Aluminium thiocyanate is used in dyeing and calico printing for three distinct purposes: as a resist for aniline black; as an addition to the ordinary alizarine red printing colour in order to resist the action of iron; and as a mordant for alizarine red instead of acetates (Lau-ber and Storck, *J. Soc. Chem. Ind.*, 1882, **1**, 359.). When applied to the last purpose thiocyanates are found to produce greater brilliance of tint and fastness on the fibre, apparently from the gradual manner in which they undergo decomposition on steaming.

Ammonium Thiocyanate.—Ammonium thiocyanate is produced by the action of ammonium polysulphide on hydrocyanic acid, or by reaction between carbon disulphide and ammonia. The latter method involves, according to Schulze (*J. prakt. Chem.*, 1883, **27**, 518), the mixing of 600 grm. of 95% alcohol with 800 grm. of ammonia (sp. gr. 0.912) and 350 to 400 grm. of carbon bisulphide, and heating the mixture for several hours under a reflux condenser. The compound is also a product of the destructive distillation of coal and is found in considerable quantities in the ammoniacal liquor and the spent purifier oxide of the gas works.

Ammonium thiocyanate crystallises in colourless anhydrous plates and is very deliquescent. 100 parts of water dissolve 122 parts of the salt at 0°, and 162 parts at 20°. In dissolving, a considerable amount of heat is absorbed.

The solid salt and its concentrated aqueous solutions absorb ammonia, and its use in certain synthetic ammonia processes has been suggested. According to Bradley and Alexander (*J. Amer. Chem. Soc.*, 1912, **34**, 15) the dry salt will absorb ammonia to give a product containing 43% of ammonia at 0° and 31% at 25°. The results of an extended study of the equilibria in the system Ammonia: Water: Ammonium thiocyanate are reported by Foote (*J. Amer. Chem. Soc.*, 1921, **43**, 1031).

When heated to about 150°, ammonium thiocyanate melts and undergoes a partial re-arrangement, with formation of thiourea— $\text{NH}_4\text{SCN} \rightleftharpoons \text{NH}_2\cdot\text{CS}\cdot\text{NH}_2$. This rearrangement is similar to that undergone by its oxygen analogue, ammonium cyanate, but is less complete than the urea re-arrangement. According to Reynolds and Werner (*J. Chem. Soc.*, 1903, **83**, **1**, 1, 24), 76% thiourea is present

at equilibrium at 170° , and only 15.2% at $148-149^{\circ}$. Gilfillan (*J. Amer. Chem. Soc.*, 1920, **42**, 2072) finds only 10.9% thiourea at 160° . The yield of thiourea is not increased by the use of higher temperatures (Reynolds and Werner), since more complex compounds, particularly guanidine thiocyanate, are formed at the expense of the thiourea as the temperatures are raised.

Barium Thiocyanate is a salt of considerable commercial importance, its principal use being for the production of aluminium thiocyanate. It crystallises with two molecules of water, both of which are lost by heating at 100° . At 15° C. 100 c.c. of the saturated solution contain 103.7 grm. of $\text{Ba}(\text{CNS})_2$, and the specific gravity of the solution is 1.705.

Calcium Thiocyanate crystallises, with great difficulty, with 3 molecules of water of crystallisation. Concentrated solutions of calcium thiocyanate have a strong action upon paper or other cellulose-containing substances, and their use has been proposed for the parchmentising of paper (Williams, *J. Soc. Chem Ind.*, 1921, **40**, 221T). Calcium thiocyanate is also recommended for use in the dyeing of acetate silk (*Amer. Dyestuff Rep.* 1926, **15**, 197).

Cuprous Thiocyanate is precipitated as a white, finely divided, amorphous precipitate when a thiocyanate is added to a solution of a cuprous salt. It is used to a limited extent as an ingredient of anti-fouling paints.

Ferric Thiocyanate.—The intensely red-coloured compound obtained in the reaction between a thiocyanate and ferric iron is ferric thiocyanate, $\text{Fe}(\text{CNS})_3$, which may be isolated in crystalline form with $1\frac{1}{2}$ molecules of water. This salt is extremely soluble in ether and may be completely extracted from an aqueous solution by shaking with ether.

Lead Thiocyanate finds limited application in the manufacture of explosives. It is a yellowish-white crystalline product, somewhat soluble in hot water, and readily soluble in water acidified with hydrochloric acid.

Mercuric Thiocyanate.— $\text{Hg}(\text{CNS})_2$, is obtained as a sparingly soluble, white, crystalline precipitate on adding a soluble thiocyanate to a strong solution of mercuric nitrate or chloride. It is soluble in excess of the precipitant and in dilute hydrochloric acid. On heating, mercuric thiocyanate ignites and swells up enormously, giving off sulphur dioxide and mercurial vapours, and leaving

a very porous, bulky, gray or brown residue. This property of the salt is made use of in the preparations of the familiar toys, "Pharaoh's Serpents."

Potassium Thiocyanate, KCNS , crystallises in colourless, anhydrous, deliquescent needles. 100 parts of water dissolve 177 parts of the salt at 0° and 217 parts at 20° , with considerable lowering of temperature.

Silver Thiocyanate.—Silver thiocyanate, AgCNS , is precipitated when a soluble silver salt is added in excess to a solution of a thiocyanate. The precipitate is curdy, resembling silver chloride in appearance, and is soluble in ammonia.

The Detection and Estimation of Thiocyanates

Detection.—Silver nitrate precipitates from solutions of soluble thiocyanates white curdy silver thiocyanate, AgCNS , insoluble in dilute nitric acid, but soluble in ammonia and in an excess of soluble thiocyanates.

Cupric sulphate produces no immediate change in a weak solution of a thiocyanate, but in a strong solution it precipitates black cupric thiocyanate, $\text{Cu}(\text{CNS})_2$, which turns white on standing. If sodium sulphite, sulphurous acid or other reducing agent is added together with the cupric solution, a white precipitate of cuprous thiocyanate, CuCNS , is immediately formed. The precipitate is insoluble in water and in salt solutions, nearly insoluble in dilute sulphuric and hydrochloric acids, but is soluble in ammonia.

When added to an acid solution of a soluble thiocyanate, ferric salts produce a deep red coloration, due to formation of the soluble, red ferric thiocyanate. This is a most delicate and characteristic reaction for ferric salts and for thiocyanates. The colour is not destroyed by boiling or by cold dilute mineral acids (distinction from acetates and formates). Fixed alkalies and ammonia precipitate brown ferric hydroxide and destroy the colour. The colour is instantly destroyed by mercuric chloride (distinction from miconates) or by excess of silver nitrate (distinction from formates and acetates). When the test is applied in presence of ferrocyanide, excess of ferric solution should be added and the liquid filtered from the precipitate of Prussian blue, when the red colour will become apparent. In presence of ferricyanide the dark coloured solution should be largely diluted.

A colour similar to that of ferric thiocyanate is obtained by addition to an acidified thiocyanate solution of a few drops of an ammonium molybdate solution followed by granulated zinc. In this case the red colour is due to formation of Mo(SCN)_4 , corresponding to MoO_2 , an intermediate stage in the reduction of molybdic acid. Long-continued action of the zinc causes the red colour to disappear, for the chloride MoCl_3 , corresponding to the oxide Mo_2O_3 , to which molybdic acid is finally reduced, gives a colourless solution with thiocyanic acid.

Estimation. Gravimetric Methods. *Oxidation and Precipitation of Barium Sulphate.*—For the gravimetric estimation of thiocyanates the compounds may be oxidised with nitric acid or other oxidising agent and the sulphuric acid so formed precipitated as barium sulphate. The action of nitric acid alone is frequently slow, particularly upon the insoluble thiocyanates, and the use for this purpose of a solution of bromine in nitric acid has been found very satisfactory.

In the analysis of soluble thiocyanates the salts are dissolved in water, and the solution of bromine in nitric acid is cautiously added until the mixture remains permanently red. The mixture is then boiled for a few minutes and is finally evaporated just to dryness after addition of a small amount of sodium chloride solution to prevent loss of sulphuric acid by volatilisation. The residue is moistened with hydrochloric acid and is again evaporated just to dryness to destroy nitric acid. It is finally taken up with water, acidified with hydrochloric acid, filtered and the sulphur precipitated with barium chloride.

Direct oxidation is, of course, not feasible in presence of other sulphur compounds, but recourse may be had to precipitation of the thiocyanate as the silver salt, followed by oxidation. Sulphides, if present, may be removed by shaking with solid lead carbonate. The solution, after removal of sulphides, is slightly acidified with nitric acid, and silver nitrate solution is added. The precipitated silver salts are filtered off and washed. They are then transferred back to the beaker in which the original precipitation was made, by aid of a jet of hot water, and the mixed salts are oxidised with nitric acid and bromine, as described above.

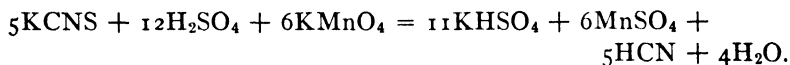
Separation and Estimation as Cuprous Thiocyanate.—The thiocyanate solution is acidified with hydrochloric acid, and a solution

of sodium bisulphite is added, followed by an excess of cupric sulphate. The white cuprous thiocyanate so precipitated, owing to its extreme state of subdivision, is exceedingly difficult to filter. The mixture is therefore allowed to stand over-night, preferably in a warm place. It may then be filtered off on a tared filter, washed, dried and weighed as cuprous thiocyanate.

Instead of the cuprous thiocyanate being weighed it may be dissolved and the thiocyanate estimated volumetrically, as described in later paragraphs.

Ferrocyanides, if also present, may be removed by a first precipitation with ferric or zinc salts, and the thiocyanate precipitated from the filtrate.

Volumetric Methods.—*Oxidation with Permanganate Solution.* Oxidation by permanganate proceeds as follows:



The method finds limited application, not only because it may be used only in absence of other oxidisable substances, but particularly because low results are apt to be obtained unless special precautions are taken.

According to Meurice (*Ann. Chim. anal. appl.*, 1920, 2, [ii], 272) the low results are due to a part of the sulphur remaining as thionic acid. He recommends the following procedure:—Add to the thiocyanate sample an excess of sulphuric acid (1:3), then add the permanganate in sufficient amount to have an excess equivalent to at least one-half that necessary for the complete oxidation of the sulphur to H_2SO_4 . Titrate back, after a definite time, with hydrogen peroxide or ferrous sulphate.

Reasonably accurate results may be secured in the permanganate titration if the permanganate solution is standardised against a thiocyanate solution of known concentration and under the same conditions of concentration, temperature, and time of titration. To obtain the best results the thiocyanate solution for titration should not be too dilute.

The permanganate titration is frequently employed in conjunction with the cuprous thiocyanate precipitation. After precipitation of the cuprous salt, as described in a previous paragraph, the precipitate is dissolved in dilute ammonia (Ronnet, *Ann. Chim. anal.*, 1911,

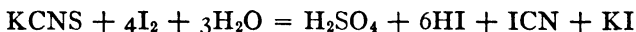
16, 336), acidified with dilute sulphuric acid, and the warm solution titrated with 0.1*N* potassium permanganate solution.

Titration with Silver Nitrate (Volhard Method).—In this familiar analytical procedure the thiocyanate solution is acidified with nitric acid, and a solution of ferric sulphate is added. This produces a deep red solution of ferric thiocyanate. *N*/10 silver nitrate is now added from a burette until the red colour is discharged. The end-point is better observed by adding an excess of silver solution and titrating back with standard thiocyanate until a permanent pink tint is produced.

In presence of ferrocyanides excess of iron solution must be added, and the liquid filtered before titrating with silver nitrate. The same plan is applicable in presence of ferricyanides if ferrous sulphate is substituted for the ferric salt. Cyanides may also be removed by ferrous and ferric salts and alkali, with subsequent acidification with dilute nitric acid, followed by filtration.

The Volhard titration may be employed in the examination of solutions containing other acid radicals whose silver salts are insoluble in nitric acid by first precipitating cuprous thiocyanate as already described, and then decomposing the insoluble salt by boiling with caustic soda or with 20% sodium carbonate solution. After filtration the solution is acidified, and the resulting solution is titrated with standard silver nitrate.

Oxidation with Iodine or Bromine.—Rupp and Schied, (*Ber.*, 1902, 35, 2191) recommend titration with iodine as shown, by the reaction.

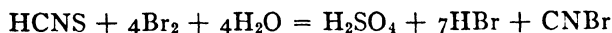


2 grm. of the soluble thiocyanate are dissolved in 1 litre of water; 10 c.c. of the solution are taken, and 25 c.c. of *N*/10 iodine solution are run in from a burette; then 1 grm. of solid sodium bicarbonate is added. The mixture is well shaken in a glass-stoppered bottle and allowed to stand for about $\frac{1}{2}$ hour. The excess iodine is titrated with *N*/10 thiosulphate solution; no starch solution may be used in titrating the iodine, as cyanogen iodide also colours the starch blue.

This method has undergone considerable modification in the hands of later investigators. Treadwell and Mayr (*Z. anorg. Chem.*, 1915, 92, 127) substitute bromine as the oxidising agent.

For the determination of HCNS, either alone or in presence of hydrochloric or hydrobromic acid, place 5 to 10 c.c. of a 0.2*N* solution in a stoppered 1 litre flask, add 50 c.c. of 0.2*N* KBrO₃ solution and 10 to 15 c.c. of 10% KBr solution, evacuate the flask under a water-pump, and add concentrated hydrochloric acid through a dropping funnel until the volume of liquid is increased one-third. Then let the flask stand 10 to 15 minutes, add concentrated potassium iodide solution through the dropping funnel, and titrate the iodine liberated by the excess of bromine with sodium thiosulphate, starch being used as indicator.

Schulek (*Z. anal. Chem.*, 1923, **62**, 337) employs bromine water according to the reaction—



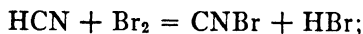
The cyanogen bromide formed is quite stable in faintly acid solution. By treatment with phenol the excess of bromine can be removed, and the CNBr can be made to react with HI thus:



and the liberated iodine titrated with thiosulphate.

To 50 c.c. of a solution containing from 0.3 to 90 mg. of HCNS in a 150 c.c. glass-stoppered flask add 5 c.c. of 20% phosphoric acid and introduce, drop-wise, enough bromine water to colour the solution distinctly yellow. Then add 30 to 40 drops of 5% phenol solution. Shake well, and, after 15 minutes, add 0.5 grm. of potassium iodide, and allow the mixture to remain in the dark for 30 minutes before titrating with sodium thiosulphate solution.

Hydrocyanic acid also reacts with bromine according to the equation—



hence when thiocyanate is to be determined cyanides must be removed by distillation with a weak acid (*e. g.* boric acid) before applying the bromine oxidation, or else the two acid radicals must be determined together, and the cyanide determined on a separate sample, thiocyanate being arrived at by difference.

Colorimetric Determination.—The characteristic coloration with ferric salts is frequently employed for the colorimetric estimation of small amounts of thiocyanates. The tests are usually made in Nessler tubes, a standard thiocyanate solution being used for the

colour standards. It is exceedingly important in making such tests that the standards contain exactly the same amount of ferric salt solution and of acid, preferably hydrochloric acid. A considerable excess of the ferric salt should be employed.

Thiocyanate analyses are rarely made in pure solutions and much ingenuity on the part of the analyst is required to select the method most applicable to the particular combination under examination. Cyanide, ferro- and ferricyanides, sulphides, sulphites, thiosulphates are commonly associated acid radicals whose behaviour in the various analytical procedures must be given careful consideration. Sulphides may be removed by shaking with solid lead carbonate, ferrocyanides by precipitation with zinc or ferric iron, ferricyanides by precipitation with ferrous salts. In presence of sulphites, thiosulphates or thio acids it is preferable to separate the thiocyanate by precipitation of the silver or cuprous salts. In general, the volumetric methods are to be employed directly only when the other constituents of the solutions are known. When these are unknown, the precipitation methods are to be recommended. The determination of the sulphur content of the silver salts insoluble in dilute nitric acid, sulphides having been previously removed, has been found most satisfactory for the examination of a wide variety of substances, notwithstanding the time required in the series of operations constituting the procedure.

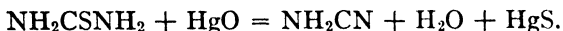
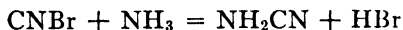
CYANAMIDE AND RELATED COMPOUNDS

The compound cyanamide, H_2CN_2 , is of interest to the analytical chemist as the free acid of the important commercial salt, calcium cyanamide. Originally prepared by reaction between a cyanogen halide and ammonia, it remained a laboratory curiosity until the discovery, in 1897, by Frank and Caro that commercial calcium carbide absorbs gaseous nitrogen at high temperatures, producing calcium cyanamide, a compound which was later shown to be a fertiliser material of merit, as well as a raw material applicable to the manufacture of a variety of other nitrogenous compounds. So rapid has been the development of this industry that, at the present time, the production of the cyanamide industry surpasses both in tonnage and in value the combined production of all the cyanogen compounds which have received treatment in preceding sections of the present volume.

In the following paragraphs we shall first discuss cyanamide and calcium cyanamide, and shall then take up those other closely related nitrogenous compounds which are obtained from cyanamide or associated with it.

Cyanamide.—Two structural formulae may be assigned to cyanamide, the amide formula, $\text{NH}_2\text{C}\equiv\text{N}$, and the diimide formula, $\text{HN}=\text{C}=\text{NH}$. Both formulae are required to explain the variety of reactions in which the compound takes part.

Besides the formation from ammonia and a cyanogen halide to which we have already referred, cyanamide may also be prepared by desulphurisation of thiourea with mercuric oxide. The reactions for the two methods are:



The sodium salt of cyanamide is produced as an intermediate product in the manufacture of sodium cyanide by the Castner process, whilst the calcium salt is produced from calcium carbide and nitrogen, as described in a later section.

Cyanamide, for laboratory uses, is most conveniently prepared from commercial calcium cyanamide by the following procedure: To 10 parts of ice-cold water add 1 part of calcium cyanamide, preferably unoled and unhydrated, and stir for half an hour, keeping the temperature of the mixture below 15° by addition of ice, if necessary. Filter the mixture and add to it 20% sulphuric acid with vigorous stirring until the mixture is just acid to methyl red indicator. Filter again, adjust the reaction of the filtrate until it is neutral or only barely acid, preferably so that the hydrogen ion concentration is between 10^{-4} and $10^{-6}N$, evaporate the solution by boiling under reduced pressure, testing the reaction occasionally and adjusting it if it is not between the desired limits. Concentrate to very small bulk, filter warm, and cool, preferably to well below zero. The crystals obtained consist of cyanamide contaminated with calcium sulphate and some dicyandiamide. To purify, extract and recrystallise from ether.

Cyanamide melts at 42° and is exceedingly soluble in water, alcohol and ether. Its crystals are stable when dry, and its aqueous solutions are also reasonably stable when cool and when of proper reaction.

The transformations undergone by aqueous solutions of cyanamide are complex and can be given only brief consideration here. In general, it may be said that the behaviour of a cyanamide solution is determined by its hydrogen ion concentration. In neutral, or only slightly acid solution, it is fairly stable and may be concentrated under reduced pressure with little or no decomposition. In alkaline solution the products depend upon the degree of alkalinity; slightly alkaline solutions yield dicyandiamide and urea, whilst strongly alkaline solutions yield urea as the principal product. Under properly regulated conditions either dicyandiamide or urea may be produced as the principal product. In strongly acidic solutions urea is produced. The reactions are



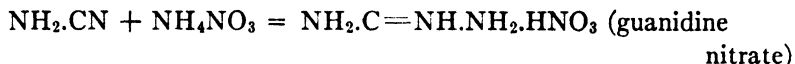
and



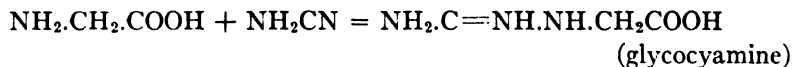
A reaction similar to that by which urea is formed occurs when cyanamide reacts with hydrogen sulphide; thiourea is formed thus:



When heated with an ammonium salt cyanamide reacts readily to form the corresponding guanidine salt, thus:



By reaction with amino acids guanidine acids are formed. Thus with glycoll, (amino-acetic acid), guanidine acetic acid or glyco-cyanamine is formed.



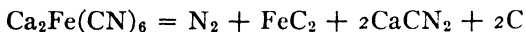
The normal cyanamides of the alkaline and the alkaline earths may be produced in the dry way, but they undergo hydrolysis in aqueous solution, with formation of the acid cyanamides and liberation of free base. Thus calcium cyanamide when dissolved in water reacts as follows:



The heavy metal cyanamides are soluble in dilute acids, but are insoluble in water and usually in ammonia. Silver cyanamide,

much employed in the analysis of cyanamide mixtures, is canary-yellow in colour and is precipitated quantitatively by addition of an ammoniacal silver solution to a solution of free cyanamide or its salts. Lead cyanamide is also yellow, the tint depending somewhat upon the conditions of precipitation. Cupric cyanamide is black, nickel cyanamide is green, and cobalt cyanamide is a rich brown, while the cyanamides of zinc and cadmium are white.

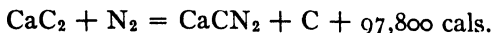
Calcium Cyanamide.—Calcium cyanamide is prepared commercially by absorption of nitrogen in hot calcium carbide, as described at some length below. It may also be prepared in several other ways. Pincass (*Chem. Ztg.*, 1922, **46**, 661) has described its production by heating calcium ferrocyanide. The reaction is—



The best results are obtained by heating for one hour at 690° . The reaction mass obtained at lower temperatures contains some ferrocyanide, while the yield of calcium cyanamide falls off at higher temperatures. Kameyama (*J. Coll. Eng. Tokyo Imp. Univ.*, 1920, **10**, 173) produces it by heating dicyandiamide and lime at 900° to 1000° for 10 minutes. The product so obtained may be enriched by heating with an excess of dicyandiamide in a current of nitrogen. Yasuda (*Tech. Rep. Tohoku Imp. Univ.*, 1924, **4**, 43) has improved this process, obtaining a product containing more than 99% CaCN_2 . The product secured by the above methods may be purified by sublimation.

Calcium cyanamide crystallises in the rhombohedral system (Warren, *Amer. J. Sci.*, 1921, **2**, 128). Its crystals exhibit very high double refraction. The m. p. of the pure crystals is $1,300^\circ$, but sublimation occurs at temperatures above $1,150^\circ$, (Ehrlich, *Z. Elektrochem.*, 1922, **28**, 529). The m. p. is lowered somewhat by lime; addition of calcium chloride has the same effect, but to a greater degree.

According to the Report on the Fixation and Utilisation of Atmospheric Nitrogen (*Nitrate Division, Ordnance Office, U. S. War Department*, 1922, p. 83), the equation for the formation of calcium cyanamide from calcium carbide is



The heat of formation at 1000° , as calculated from the Clausius equation, is 66,000 cal. The equilibrium pressure of nitrogen over

mixtures of calcium carbide, calcium cyanamide and carbon was extrapolated to one atmosphere at $1,366^{\circ}$ in one experiment with 60% carbide, and to 1490° with 80% carbide.

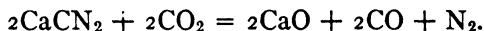
Franck and Hochwald (*Z. Elektrochem.*, 1925, **31**, 581) by a direct determination of the heat of nitrification arrived at the value 72,000 cal. for the heat of formation of calcium cyanamide from calcium carbide and nitrogen, a value which is confirmed by Kameyama and Oka (*Proc. Imp. Acad. Japan*, 1927, **3**, 161).

Ehrlich (*loc. cit.*) gives a series of figures for the decomposition pressure obtained from 90 to 95% CaCN_2 at temperatures between 1000° and 1200° .

Temp. $^{\circ}\text{C}$.	Nitrogen pressure, mm. Hg.
1,000	2.5
1,100	14
1,140	32
1,190	72

He states that the presence of free carbon, as in commercial calcium cyanamide, raises the decomposition pressure.

Calcium cyanamide is decomposed immediately by cold water, with formation of acid calcium cyanamide, $\text{Ca}(\text{HCN}_2)_2$, and free lime. With carbon dioxide decomposition occurs at temperatures of 700° or higher. Nitrogen is set free according to the reaction—



Carbon monoxide does not react at temperatures up to 1000° . Hydrogen causes liberation of small amounts of ammonia and of hydrocyanic acid.

The calcium cyanamide of commerce, one of the important products of electrochemical industry, is prepared on a very large scale by the absorption of nitrogen in hot calcium carbide. The product so obtained finds application as a fertiliser, as a raw material for the manufacture of ammonia and ammonium salts, and as an intermediate in the production of a variety of nitrogenous compounds.

In the *process of manufacture*, calcium carbide, prepared by the electrothermic smelting of lime and coke, is finely ground and is then exposed to the action of gaseous nitrogen, the mass being locally heated to about 1000° to start the reaction. Once begun, the nitrogen absorption continues without further application of heat. When absorption is complete the product,

which has sintered into a cake or "pig" during the nitrification, is withdrawn from the oven, allowed to cool, and is finally ground.

The furnace product, known as crude calcium cyanamide, contains from 19 to 24% of fixed nitrogen, the nitrogen content depending largely upon the grade of the calcium carbide from which it was produced. The ground material is used as a starting material for the production of certain chemical products, notably for calcium cyanide. In addition to calcium cyanamide, free lime, and graphite, the crude furnace product contains a small amount of residual calcium carbide which must be removed from the product intended for use in ammonia production and in the fertiliser trade. Calcium cyanamide intended for the former use is treated with an amount of water just sufficient to eliminate residual carbide. The product so treated is known as "minimum hydrated." Material for fertiliser uses is treated with a larger proportion of water, together with from 2 to 4% of an oil to reduce dusting. The oil used in the United States is a mineral oil of high-flash-test. Tar oils are much used in Europe. The oiled product is sold on the American market under the proprietary name "Cyanamid."

The following is a representative analysis of the "Cyanamid" sold in the United States:—

Total nitrogen, 20.48% (equivalent to 24.90% NH_3 or 58.5% CaCN_2); total calcium, 40.30%; free carbon, 11.34%; oil, 4.53%; silica and insoluble, 2.90%; $(\text{Fe} + \text{Al})_2\text{O}_3$, 1.56%; MgO , 0.32%; sulphur, 0.58%.

The commercial material is a grayish-black powder with an alliaceous odour. This slight, but characteristic, odour can usually be observed in mixtures in which calcium cyanamide has been used.

The statement is frequently encountered in the literature that calcium cyanamide loses its nitrogen on standing. This erroneous statement is to be explained by the fact that the material absorbs moisture and carbon dioxide from the air and so gains in weight. This gain in weight is naturally reflected in a decrease in the percentage of nitrogen, although the actual amount of nitrogen remains the same. Jacob, Krase and Braham (*Ind. Eng. Chem.*, 1924, 16, 684) have made a careful study of calcium cyanamide in storage. They conclude that properly hydrated and oiled material, stored in 45 kilo bags over a period of six months, suffers practically no

change when stored under normal conditions of temperature and humidity.

Uses of Calcium Cyanamide.—By far the largest proportion of the calcium cyanamide of the world is employed in agriculture by direct application to the soil. When properly applied, hydrolysis of the cyanamide takes place, with production of urea, and the calcium component is liberated as calcium hydroxide. The material is thus to be considered an organic ammoniate. Owing to its high alkalinity calcium cyanamide is particularly indicated for use in soils inclined towards acidity.

Although direct application is almost universally practised in Europe, where by far the largest percentage of the world's calcium cyanamide is made and used, this method is less commonly employed in the United States, where agricultural practice does not favour the direct application of any single fertiliser ingredient, preferring instead to employ mixed fertilisers containing two and, more often, three of the chemical elements requisite for plant nutrition. Cyanamide is thus used in the United States almost exclusively in ready-mixed goods.

Calcium cyanamide is a valuable constituent of mixed fertilisers, its high alkalinity making it an excellent neutralising and conditioning agent. Its application, however, is limited by its alkalinity, for, if used in large amounts, it may render insoluble, or "revert," some of the soluble phosphate of the mixture. This alkalinity places a practical limit on the amount of calcium cyanamide which can be used with safety of 40 to 60 pounds per ton of fertiliser mixture.

A second limitation on the amount of calcium cyanamide which can be compounded into a ton of mixed goods arises from the fact that when large amounts are used, dicyandiamide, the polymer of cyanamide may result. This compound is agriculturally undesirable, being without value as a plant food and toxic to soil bacteria when present in large amounts.

The limitations above set forth apply particularly to mixtures in which cyanamide is the only organic ammoniate present. When other organic fertilisers are used with the cyanamide, such as tankage, ammoniated base, fish scrap and the like, substantially larger amounts of cyanamide may be used with safety, the quantity usually being determined by actual experiment.

Besides its use directly, there has been considerable interest in the preparation from calcium cyanamide of various fertiliser compounds in which the cyanamide nitrogen has been converted into some other form, usually into urea. The excess lime, which at present limits its use, is thus eliminated or reduced. Several such preparations are now on the European market.

A second important use for cyanamide is as a raw material for ammonia production. The calcium cyanamide is mixed with water and is heated with steam in presence of a small amount of free alkali. Conversion to ammonia is practically quantitative, the ammonia being liberated as a gas which may be used for production of ammonium salts, aqua ammonia, or for catalytic oxidation to nitric acid.

The production of cyanides from calcium cyanamide has already been discussed in the section on the manufacture of calcium cyanide.

Finally, smaller amounts of calcium cyanamide find application in the manufacture of miscellaneous nitrogenous compounds, such as dicyandiamide, urea, thiourea, etc.

Dicyandiamide.—Dicyandiamide, formed by the combination of two molecules of cyanamide, is best represented by the cyanoguanidine formula of Bamberger, $\text{NH}_2\text{C}:(\text{NH})\text{NH}\cdot\text{CN}$. The mechanism of its formation from cyanamide has been studied by Grube and Krüger, who assume reaction between free cyanamide and cyanamide ion. As stated elsewhere, the polymerisation is most rapid in presence of weak alkalies.

Dicyandiamide may be considered a cyanamide in which one hydrogen atom has been replaced by the guanyl group. $\text{NH}_2\cdot\text{CNH}$ —; dicyandiamide is thus guanyl cyanamide. Its reactions recall those of cyanamide. Thus it combines with ammonia to form biguanide, a reaction analogous to the combination of cyanamide with ammonia to form guanidine. With hydrogen sulphide it forms guanylthiourea by a reaction similar to that by which thiourea is produced from cyanamide. It is hydrolysed by strong acids to form the substituted urea, guanylurea.

Aqueous solutions of dicyandiamide may be concentrated with little or no decomposition by boiling at atmospheric pressure. Crystallised from water it separates in broad lustrous plates whose melting point is 205° . Its solubility in water and in ethyl and methyl alcohol is shown in the following table.

THE SOLUBILITY OF DICYANDIAMID

Temperature, ° C.	Grams dicyandiamide dissolved by 100 grams of solvent		
	Water	95 % ethyl alcohol	99 % methyl alcohol
0	1.27	0.937
1.0	3.46
13.0	2.26	1.26
20.0	4.88
25.0	4.13
26.4	1.70
29.5	5.63
35.0	2.26
39.0	7.45
39.9	7.76
49.8	11.80
49.9	3.30
50.0	9.45
60.1	18.75	4.13
63.0	12.35
74.5	32.58

All the solubility data for water and ethyl alcohol are due to Braham and Hetherington (*J. Ind. Eng. Chem.*, 1923, **15**, 1060), with the exception of the values for water and ethyl alcohol at 13°.

Dicyandiamide is practically insoluble in ether, and this difference from cyanamide is utilised for the separation of the two compounds. According to Braham and Hetherington, 0.01 grm. dicyandiamide dissolves in 100 grm. of ether at 13° and 0.0026 grm. at 35.3°.

Although a compound of high nitrogen content, it is the consensus of opinion among fertiliser experts that dicyandiamide is without agricultural value. At high concentrations it is injurious, owing to a poisoning action exerted upon the nitrifying bacteria of the soil. A discussion of the action of dicyandiamide in the soil, together with a bibliography, may be found in an article by Jacob, Allison and Braham (*J. Agric. Res.*, 1924, **28**, 37).

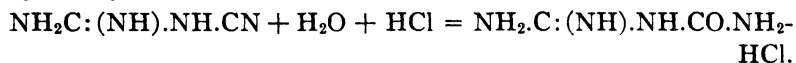
Limited quantities of dicyandiamide are employed in the production of salts of guanidine and guanylurea. A synthesis of veronal from dicyandiamide has been described (*Z. angew. Chem.*, 1914, **27**, 725).

Urea.—Although urea is formed by a large number of chemical and biological reactions, familiar to the organic chemist, its production from cyanamide is one of the most economical methods known. In strongly acid aqueous solutions cyanamide is very

rapidly converted into urea; the same change occurs, although less rapidly, in strongly alkaline solutions. The transformation also occurs when calcium cyanamide is applied to the soil or is introduced into many organic and inorganic mixtures, and it is this reaction which is responsible for the agricultural response secured from cyanamide applications.

It will be unnecessary to introduce here any discussion of the chemical and physical properties of urea, but much attention will be given in later sections to its identification and determination, particularly in mixtures containing cyanamide or derived from calcium cyanamide, since such analyses have become exceedingly important in the examination of modern fertiliser mixtures.

Guanylurea.—The sulphate or chloride of guanylurea is conveniently produced by the action of the corresponding diluted acid upon dicyandiamide. The reaction is—



The sulphate is usually prepared, owing to the ease with which it may be recovered from aqueous solution by crystallisation. The dihydrate of guanylurea sulphate, $(\text{C}_2\text{H}_6\text{ON}_4)_2\text{H}_2\text{SO}_4 \cdot 2\text{H}_2\text{O}$, is soluble in water and in alcohol. Its crystals effloresce in dry air and lose all their water when heated to 110° . Guanylurea sulphate is also produced when solid calcium cyanamide is added to strong sulphuric acid.

By decomposition of the sulphate with barium hydroxide the free base can be secured. This crystallises from dilute alcohol with one molecule of water of crystallisation. Guanylurea is a strong base and absorbs carbon dioxide from the air. It is decomposed by heating with barium hydroxide into carbon dioxide, ammonia and urea.

The characteristic salts used for the identification and the estimation of guanylurea are the picrate and the nickel compound. Both are described in later paragraphs.

No commercial use of importance has thus far been developed for the salts of guanylurea. It probably has some slight agricultural value, but is not as effective in this respect as urea. It is not toxic to soil bacteria except at very high concentrations. The use of guanylurea salts, notably the perchlorate, has been proposed in explosives.

Guanylurea is frequently styled dicyandiamidine in earlier literature.

Guanidine.—Guanidine thiocyanate was formerly prepared by heating ammonium thiocyanate. As stated elsewhere, thiourea is the first product of the rearrangement, but at elevated temperatures guanidine thiocyanate is produced. This rearrangement has been studied by Krall (*J. Chem. Soc.*, 1913, 103, 1378). The best single equation for the entire change is said to be



Preparation of guanidine salts from ammonium thiocyanate may now be considered of historic interest only, since its preparation from cyanamide or from dicyandiamide is much simpler. Three methods of preparation merit attention.

1. By the action of strong mineral acids on dicyandiamide.
2. By fusing dicyandiamide with ammonium salts.
3. By heating cyanamide with ammonium salts.

Preparation by Method 1 is described by Levene and Senior (*J. Biol. Chem.*, 1916, 25, 623). Guanylurea salts are first produced and are subsequently decomposed, liberating carbon dioxide and forming the salts of ammonia and of guanidine.

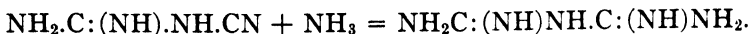
Process 2 may be conducted either by fusion of the dry salts or by heating in an autoclave in presence of small amounts of water. Both processes yield satisfactory results, although the latter method is to be preferred on account of its somewhat greater safety. By heating dicyandiamide with ammonium nitrate yields of 85% of guanidine nitrate may be obtained.

Blair and Braham have recently described the production of guanidine salts by heating aqueous solutions of free cyanamide with an ammonium salt (*J. Ind. Eng. Chem.*, 1924, 16, 848). The yields are excellent.

The properties of guanidine and its salts need not be discussed here. The picrate is employed for its identification and determination.

The salts of guanidine find a limited application in the manufacture of explosives. Much attention has recently been given to nitroguanidine as a constituent of explosive mixtures. It is conveniently prepared from guanidine nitrate by treatment with sulphuric acid.

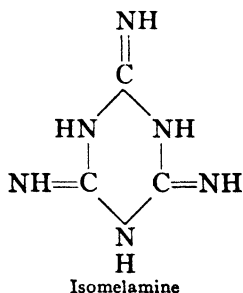
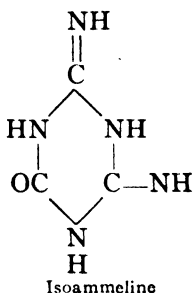
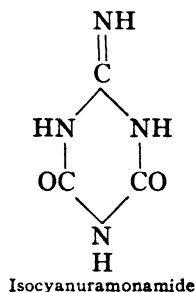
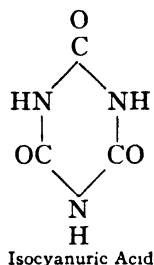
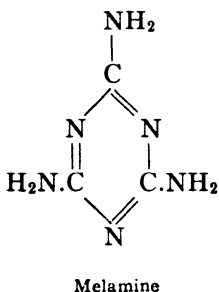
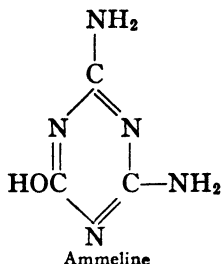
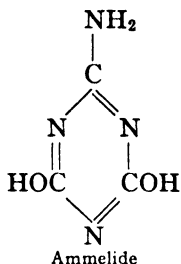
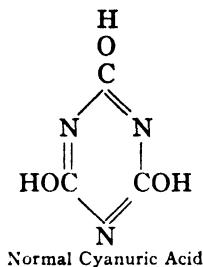
Biguanide.—Biguanide, although of comparatively rare occurrence in cyanamide mixtures, is, nevertheless, occasionally encountered. The sulphate of biguanide is conveniently prepared by heating dicyandiamide with ammoniacal copper sulphate. The copper salt is crystalline and is easily separated and the free base recovered from it by treatment with hydrogen sulphide. The reaction for the formation of biguanide is



Biguanide is identified by means of its picrate and its nickel salt. Since these same precipitants are used for the identification of guanyllurea, the possibility of error in identification exists. Blair and Braham (*J. Amer. Chem. Soc.*, 1922, **44**, [ii], 2347) discuss the peculiarities of the picrate of biguanide. They point out that while the picrate of guanidine (and also of guanyllurea) forms almost immediately, even at low concentrations, yielding fine light yellow crystals, biguanide, even in fairly concentrated solutions, is only very slowly precipitated as long thin rods. Precipitation is not at all quantitative; in dilute solutions no precipitate at all is secured.

Miscellaneous Related Compounds.—*Cyanurea*, $\text{NH}_2\cdot\text{CO}\cdot\text{NHCN}$, is produced by heating dicyandiamide with barium or calcium hydroxide. Its characteristic compound is a copper salt, $(\text{C}_2\text{N}_3\text{H})\text{CuO}\cdot 2\text{H}_2\text{O}$, which is prepared by addition to its solutions of a copper chloride solution, filtering, and allowing the filtrate to crystallise.

Of the amides of cyanuric acid, *melamine* is the most important. It is formed in small amount when suspensions of calcium cyanamide are boiled with water. Melamine also yields a precipitate with picric acid which has a characteristic appearance. Instead of the granular precipitates yielded by guanyllurea and guanidine, melamine yields a bulky mass of felted needles. Melamine has the same empirical formula as cyanamide and dicyandiamide and is not an uncommon impurity in commercial dicyandiamide. There are two melamines, normal melamine and isomelamine, derived respectively from cyanuric acid and from the hypothetical isocyanuric acid. The relation of these melamines to each other and to *ammelide* and *ammeline*, two other less common cyanamide decomposition products, is shown in the following arrangement from Richter, *Organic Chemistry*, I, 473 (1922).



The Detection and Estimation of Cyanamide and Its Related Compounds

Detection.—The increase in the technical application of calcium cyanamide which has taken place within the last decade has been rapid. Besides its extensive use in fertiliser mixtures, it is a useful starting material for the production of such important compounds as urea, thiourea, dicyandiamide and guanidine. Its place in the preparation of ammonia and of cyanides is equally well established. As a result of this widening of the applications of calcium cyanamide, the analytical chemist may be called upon to examine many mixtures which either contain cyanamide or have been prepared from

it. His task in this respect is not easy, because, owing to its reactivity, unchanged cyanamide seldom persists for any length of time, and, more often than not, the use of cyanamide in the preparation of a given mixture must be established by identification of one or other of its known reaction products.

In the following discussion of the qualitative chemistry of the cyanamide group we will first briefly summarise the qualitative tests usually relied upon for such identifications, and will then present a formal qualitative scheme which has been found applicable to the examination of the simpler fertiliser mixtures and to the more commonly encountered chemical mixtures. This scheme provides for the identification of nine nitrogenous forms when present in practically any combination. These nine forms, which include the more common members of the cyanamide group, were selected, not because they are all closely related to cyanamide in a structural sense, but rather because of their commercial relationships.

Cyanamide is usually identified by its silver salt, insoluble in water and in ammonia, but soluble in nitric acid. This test is not applicable in presence of thiourea. Thiourea occurs much more frequently in cyanamide mixtures than is commonly suspected, owing to the ease of reaction between cyanamide and sulphide sulphur, the latter being of common occurrence in chemical mixtures.

For the identification of dicyandiamide, the silver salt, insoluble in very dilute nitric acid, is a valuable aid. Details of this test are given in the qualitative scheme below. The formation of silver picrate monocyanoguanidine as described in the quantitative method of Harger, and conversion into guanylurea and identification of the latter, are useful supplements to the silver nitrate test. Both reactions are described in detail in the section on the estimation of the cyanamide compounds.

Urea is best identified by the urease test as described in a later section. As a supplementary procedure the method of Fosse (*Ann. Chim.*, 1916, 6, 13) may be helpful. The urea solution is treated with 3.5 times its volume of glacial acetic acid and then with half its volume of a methyl alcohol solution of xanthidrol (10%). A white crystalline precipitate of xanthyl urea results. The test is of doubtful value in presence of cyanamide.¹

¹ Attention has recently been directed to the fact that xanthidrol is so unstable that commercial samples are often only the decomposition products (xanthone and xanthane), which do not give decomposition products with urea (Kny-Jones and Ward, *Analyst*, 1929

Guanylurea is identified by its picrate and by its nickel salt. The picrate test is, unfortunately, not specific, since guanidine and the less common melamine and biguanide also yield picrates. The picrates of melamine and biguanide differ in appearance from those of guanylurea and guanidine. The picrates of guanylurea and guanidine are not sufficiently dissimilar to permit of differentiation; for identification of guanylurea recourse must be had to the nickel salt. Guanidine does not yield an insoluble nickel salt. Biguanide yields an insoluble nickel salt, but is a form rarely encountered.

Salts of guanidine, like salts of ammonia, yield a precipitate with Nessler's reagent. The test is delicate, but can be applied only in absence of ammonia. In absence of guanylurea, guanidine salts may be identified by fusing with urea at 160° ; guanylurea is formed which may be identified by means of the nickel salt. The test ordinarily applied is that with alcoholic picric acid. Guanidine picrate is soluble in 2,630 parts of water at 9° , and difficultly soluble in alcohol and ether. The precipitation of the picrate is practically quantitative in pure solutions, but in presence of other salts this is not always the case.

Biguanide yields a characteristic picrate and a nickel salt.

Melamine yields a picrate of very characteristic appearance easily recrystallised from warm water.

Qualitative Scheme for Detection of Cyanamide and Related Compounds

The following scheme (Buchanan, *J. Ind. Eng. Chem.*, 1923, **15**, 637) provides for the identification of—

Ammonia and its salts
Cyanamide
Hydrocyanic acid and its salts
Dicyandiamide
Guanidine and its salts
Guanylurea and its salts
Thiocyanic acid and its salts
Thiourea
Urea.

54.). Hence the reagent should always be freshly made for the test by reducing xanthone, which is quite stable, by means of alcoholic sodium amalgam. The alkaline alcoholic solution is poured into water, the precipitate collected on a filter and washed, partly dried at the ordinary temperature, and re-dissolved in alcohol for use in the test for urea. Xanthidrol is more stable in solution than in the solid condition, and it is useless to keep it in that form.

It is assumed that the mixtures under examination contain no nitrogenous compounds other than those listed. Provision is made for the interference of the inorganic radicals most commonly found in commercial mixtures. The scheme consists of three parts.

1. Preliminary tests for the Most Common Interfering Radicals: Sulphide and phosphates are commonly encountered in commercial mixtures; both must be eliminated before the qualitative tests are applied.

2. Preparation of Solution for Analysis.

3. Identification of Nitrogenous Forms: Tests and such comments as seem necessary for their intelligent application are given. The procedures contain the analytical directions; comments are reserved for the notes.

1. Preliminary Tests

Sulphide Sulphur.—Shake a small portion of the material with cold water, filter if necessary, and add to the clear filtrate a little lead carbonate which has previously been ground to a pulp with water. Shake for 1 minute. (Blackening of the lead carbonate indicates presence of *inorganic sulphide sulphur*.)

Phosphate.—Filter the lead carbonate mixture obtained in the test for sulphide sulphur, acidify the clear filtrate with nitric acid, add a little solid ammonium nitrate, heat to 60° , and add a few c.c. of ammonium molybdate solution (yellow precipitate: presence of *phosphate*).

Note:—This qualitative scheme is intended for use particularly in the examination of fertiliser mixtures in which water-soluble phosphate is an important constituent. Provision for the removal of this interfering radical is made in a later paragraph.

2. Preparation of Solution

Absence of Phosphate.—If the material is completely soluble and if sulphide sulphur is absent, dissolve 5 grm. in about 150 c.c. of cold water. If it is not completely soluble or if inorganic sulphides are present, grind 5 grm. in a small mortar with 50 c.c. of cold water, add sufficient caustic soda to make the reaction alkaline or neutral to litmus, if it is not already so, and then add 2 grm. of lead carbonate previously ground to a pulp with water. Shake the mixture for

several minutes, filter, and make up the filtrate and washings to a volume of about 150 c.c. In either case make the resulting solution neutral to litmus, adding dilute caustic soda or dilute sulphuric acid as required. Filter off and reject any precipitate that may be formed. Dilute the solution to a volume of 250 c.c., mix thoroughly, and keep in a cool place.

Presence of Phosphate.—Weigh a 10 grm. sample into a mortar, add about 50 c.c. of water and then sufficient milk-of-lime to precipitate the phosphates completely, and grind with a pestle. Pour the mixture into a 250 c.c. flask and add lead carbonate. If sulphides are present, dilute to 250 c.c. and shake. Pour through a dry filter and test for complete removal of phosphate. Neutralise 125 c.c. of the filter to litmus with dilute sulphuric acid, filter if necessary, and dilute to 250 c.c.

Notes.—(1) A weighed sample is used to permit a rough estimate of the amounts of the various nitrogenous forms present. In the examination of mixtures known to be low in nitrogen, as for example many fertiliser mixtures, a larger sample may be taken. All of the tests will detect 0.5 mg. of nitrogen per c.c. of solution, and nearly all will detect 0.2 mg. per c.c.

(2) The extraction should be made in the cold, and the solution so prepared should be used on the day it was prepared to avoid change of the nitrogenous forms, particularly of the cyanamide.

(3) The final solution is made neutral to litmus, because cyanamide solutions are most stable under such conditions.

(4) It is assumed that all the nitrogenous forms are soluble in water. The scheme does not provide for insoluble forms, such as the metallic salts of cyanamide, of hydrocyanic or of thiocyanic acid. Certain less common decomposition products of cyanamide, ammeline, for example, are insoluble in water and are eliminated at this point.

3. Identification of Nitrogenous Forms

Test 1. Ammoniacal Silver Nitrate.—To a 10 c.c. portion of the solution add 1 c.c. of strong ammonia and 1 c.c. of 10% silver nitrate. Shake for 1 minute. (Bright yellow precipitate: presence of *cyanamide*. Black precipitate: presence of *thiourea*.)

Notes.—(1) Thiourea is desulphurised by ammoniacal silver nitrate, and a precipitate of silver sulphide and silver cyanamide is

produced. Silver cyanamide is bright canary-yellow. If the precipitate is brown or black, thiourea is present, provided the inorganic sulphides were properly removed during the preparation of the solution. If thiourea is present, pass on to Test 2; if absent, omit Test 2 and pass on to Test 3.

(2) The volume of the precipitating reagent recommended in this and in the subsequent tests is the quantity necessary to insure detection of the nitrogenous constituent when present in small amounts. With larger amounts, larger quantities of reagent may be required for complete precipitation. Where a separation is to be made the analyst is expected to test for complete precipitation.

(3) When large quantities of cyanide and thiocyanate are present, 1 c.c. of strong ammonia may be insufficient to keep their silver salts in solution, but, since these salts are white, their precipitation will not prevent the detection of either cyanamide or thiourea. The ammonia addition should not be increased, as this would lessen the delicacy of the cyanamide test.

(4) Guanylthiourea, a less common form, is identified as thiourea by this test.

Test 2. *Cyanamide in Presence of Thiourea*.—To a 10 c.c. portion of the solution add 1 c.c. of saturated lead acetate solution and then 1 c.c. of strong ammonia. Shake for 1 minute. (Yellow precipitate: presence of *cyanamide*; white precipitate: absence of *cyanamide*.)

Notes.—(1) Ammoniacal lead acetate does not desulphurise thiourea. Lead cyanamide is similar in appearance to silver cyanamide, but the full yellow colour develops more slowly. When very small amounts of cyanamide are present, several minutes may be required for the appearance of the yellow colour.

(2) Tests 1 and 2 are given precedence, because, on account of its instability, mixtures containing cyanamide must be handled with special precautions. In absence of cyanamide many of the subsequent tests are simplified.

Test 3. *Acid Silver Nitrate*.—To a 10 c.c. portion of the solution add 3 drops of concentrated nitric acid and 1 c.c. of 10% silver nitrate. Shake for 1 minute, and, if no precipitate forms, cool in an ice bath for 30 minutes, with occasional shaking. (White precipitate: presence of compounds of Group 1 or of a chloride. No precipitate: absence of compounds of Group 1.)

Group 1. Precipitated by acid silver nitrate

Cyanide
Dicyandiamide
Thiocyanate
Thiourea

Group 2. Not precipitated by acid silver nitrate

Ammonia
Cyanamide
Guanidine
Guanylurea
Urea

Notes.—(1) If acid silver nitrate yields no precipitate, all compounds of Group 1 are absent. Omit Tests 4, 5, 6 and 7. If a precipitate is obtained, one or more compounds of Group 1 may be present, or the precipitate may be that of a halogen. The members of Group 1 are now identified by the following tests: thiocyanate, by Test 4; cyanide by Test 5; and dicyandiamide by Tests 6 and 7. Thiourea has already been identified in Test 1.

(2) Silver cyanide and thiocyanate are very insoluble in dilute nitric acid and are precipitated rapidly and completely. Silver dicyandiamide, even when present in considerable amounts, forms slowly, separating in voluminous semi-transparent flocks, or frequently in long needle-shaped crystals. Its appearance is characteristic, and once seen it is easily recognised. It is much more soluble in warm than in cold solutions, and thorough chilling, as in an ice and salt bath, may be necessary to produce the precipitate when dicyandiamide is present in small amounts.

(3) Since commercial materials usually contain sufficient chloride to yield a precipitate at this point, a positive result has little significance. If the test is negative, however, the examination is materially simplified.

Test 4. Thiocyanate.—To a 10 c.c. portion of the solution add 2 drops of concentrated hydrochloric acid and then 5 drops of 10% ferric chloride solution (red coloration: presence of *thiocyanate*).

Test 5. Cyanide.—To a 10 c.c. portion of the original solution add 1 c.c. of 10% caustic soda and 5 drops of a saturated solution of ferrous ammonium sulphate. Heat for a few minutes and then add 2 drops of 10% ferric chloride solution, followed by dilute hydrochloric acid, drop by drop, until the hydroxides of iron are completely dissolved. Pour the mixture through a small filter and wash with water. (Blue precipitate: presence of *cyanide*.)

Test 6. Dicyandiamide in Absence of Thiourea.—Repeat Test 3. If a precipitate forms as soon as the silver nitrate is added, allow it to settle, and then add more silver nitrate, drop by drop, until no further precipitate is formed. Heat the mixture to 60° on a water-

bath and then filter. Add 5 drops of the silver solution and cool in an ice-bath, with occasional shaking, allowing the mixture to stand for at least 30 minutes. (White precipitate which separates on cooling the solution: presence of *dicyandiamide*.)

Notes.—(1) The silver compound of dicyandiamide is much more soluble in warm than in cold water, and an approximate separation may be made from cyanide, thiocyanate and chloride, the silver salts of which are insoluble in both hot and cold solutions. The appearance of silver dicyandiamide is described in Note 2 under Test 3.

(2) The temperature of the mixture should be raised to 60° without delay, and the heating should not be prolonged, since dicyandiamide may be converted to guanylurea by the nitric acid.

Test 7. *Dicyandiamide in Presence of Thiourea.*—To a 10 c.c. portion of the solution add 3 c.c. of strong ammonia and 3 c.c. of 10% silver nitrate. Shake until the precipitate is coagulated and then add silver nitrate, drop by drop, until no further precipitate forms. Filter off the black precipitate and add to the clear filtrate 5 drops of silver solution as a test for complete precipitation. If no permanent precipitate is produced by this last silver addition, add nitric acid, drop by drop, until the solution is just acid and then 5 drops in excess, keeping the mixture cold during the addition. If no precipitate forms at once, cool for 30 minutes in an ice-bath with frequent stirring (no precipitate: absence of *dicyandiamide*.) If a precipitate is obtained add silver nitrate, drop by drop, until no further precipitate is formed, heat to 60°, and proceed as in Test 6.

Note.—Ammoniacal silver precipitates silver sulphide from thiourea, leaving cyanamide in solution if insufficient silver is used, or precipitating it if an excess of silver is present. After filtering off the precipitate obtained with ammoniacal silver, the solution is in proper condition for the application of Test 6.

Test 8. *Picric Acid.*—To a 10 c.c. portion of the solution add 1 c.c. of alcoholic picric acid and stir for 5 minutes, using a stirring rod and scratching the sides of the container. (Yellow precipitate: presence of *guanylurea* or *guanidine*, or both.)

Notes.—(1) If test 8 is negative, both *guanylurea* and *guanidine* are absent. Omit Tests 9 and 10 and pass on to Test 11. If Test 8 is positive, *guanylurea* or *guanidine*, or a mixture of the two, may be present. Proceed to Tests 9 and 10.

(2) The alcoholic picric acid solution is prepared by dissolving 6 grm. of picric acid in 100 c.c. of 95% alcohol.

(3) The analyst should familiarise himself with the appearance of the picrates of guanylurea and guanidine, since other precipitates are sometimes obtained when the reagent is added. Thus picric acid itself may be precipitated from concentrated salt solutions, particularly if the solutions are cold. The addition of alcohol alone may produce a precipitate, and this possibility should be examined by the addition of 1 c.c. of alcohol in a blank test.

(4) It is assumed that the solutions under examination contain no nitrogenous compounds other than the nine listed. With care, however, the scheme may be applied to the examination of fertiliser mixtures containing ammoniated base or similar products of the acid hydrolysis of proteins. Since ammoniated base usually gives a flocculent precipitate with picric acid, it is necessary to defecate the solution with basic lead acetate before applying the test. Mixtures containing cottonseed meal also sometimes give a gelatinous precipitate with picric acid. This may be eliminated by treatment with basic lead acetate. Guanylurea and guanidine are not removed by basic lead acetate. In general, then, if the precipitate in Test 8 is gelatinous or flocculent, and if a commercial fertiliser mixture is under examination, repeat the test on a fresh portion of the solution after defecation with basic lead acetate.

(5) Several of the less common decomposition products of cyanamide, notably melamine, biguanide and guanylthiourea, also yield precipitates of characteristic appearance with picric acid.

Test 9. *Nickel Test for Guanylurea.*—To a 25 c.c. portion of the solution add 5 c.c. of nickel reagent and then 10 c.c. of 10% sodium hydroxide solution. Leave in an ice-bath for 1 hour, scratching the sides of the container occasionally with a stirring rod. Pour through a small filter and wash once with water, keeping filtrate and washings separate. (Orange crystalline precipitate: presence of *guanylurea*.) Use the filtrate for Test 10. Reject the washings.

Notes.—(1) Guanidine yields no precipitate with nickel. If Test 9 is positive, pass on to Test 10. If negative, the positive result obtained in Test 8 is considered sufficient for the identification of guanidine.

(2) The nickel reagent is made up as follows: Dissolve 10 grm. of nickel sulphate and 30 grm. of citric acid in 150 c.c. of water and add 50 c.c. of strong ammonia.

(3) When considerable amounts of guanylurea are present the formation of the crystalline precipitate is preceded by a colour change from blue to yellow, which occurs as soon as the caustic soda is added. This colour is not to be confused with the blue-green colour change which always occurs when caustic soda is added to the nickel reagent.

(4) The crystalline precipitate of nickel guanylurea forms slowly, and the test should not be considered negative until the mixture has stood for at least an hour. A flocculent precipitate which sometimes develops on long standing must not be confused with the characteristic orange crystals of nickel guanylurea.

(5) Biguanide also yields an insoluble nickel salt.

Test 10. *Guanidine in Presence of Guanylurea.*—To the filtrate from Test 9 add concentrated hydrochloric acid, drop by drop, until it is just acid to litmus, and then add 1 or 2 drops of ammonia and 2 c.c. of alcoholic picric acid. Cool in an ice-bath with occasional stirring. (Yellow crystalline precipitate: presence of *guanidine*.)

Note.—It is, of course, essential that enough nickel reagent to precipitate the guanylurea completely should be used, and that sufficient time for complete precipitation should be allowed. The filtrate from the nickel guanylurea should have a green colour, showing the presence of an excess of nickel.

Test 11. *Ammonia in Absence of Cyanamide.*—To a 10 c.c. portion of the solution add 1 grm. of magnesium oxide which has been ground to a pulp with water, and heat to boiling. Identify *ammonia* by its odour or by use of litmus paper.

Note.—If cyanamide is present, it must be removed as in Test 12. If the result of Test 11 is uncertain, repeat it as follows: Dilute a 50 c.c. portion of the solution with 100 c.c. of water, add 2 grm. of magnesium oxide, and boil the mixture in an ammonia-distilling apparatus for just 15 minutes, absorbing the distillate in water. Add 2 drops of methyl red indicator, and titrate the liberated ammonia with 0.1*N* sulphuric acid.

Test 12. *Ammonia in Presence of Cyanamide.*—To a 25 c.c. portion of the solution add 5 c.c. of 10% silver nitrate solution and then 5 c.c. of 10% caustic soda. Shake to coagulate the precipitate.

filter and wash twice with cold water. Add to the filtrate 5 grm. of magnesium sulphate dissolved in a little water, dilute to about 150 c.c. and heat to boiling. Identify *ammonia* by the odour, by use of a piece of litmus paper, or by distilling into water, as directed in the note of Test 11.

Note.—Cyanamide is completely precipitated by silver nitrate in caustic soda solution. The addition of the magnesium sulphate to the resulting filtrate, which contains an excess of caustic soda, precipitates magnesium hydroxide and gives the conditions of Test 11.

Test 13. *Urea.*—To a 25 c.c. portion of the solution add 1 drop of methyl red indicator, make exactly neutral with 0.02*N* sulphuric acid or caustic soda as required, and then add just enough of the acid to impart a distinct pink colour. Suspend 0.1 grm. of soya bean flour in 10 c.c. of water by vigorous shaking for several minutes. Add a drop of methyl red indicator and make the suspension exactly neutral with 0.02*N* acid or alkali. Filter the soya bean suspension, and add the extract to the solution under examination. Allow the mixture to stand for 1 hour in a warm place. (Colour change, pink to yellow: presence of *urea*.) Roughly determine the quantity of urea present by titrating the liberated ammonia with 0.02*N* sulphuric acid.

Notes.—(1) The soya bean flour is prepared by peeling and drying the soya beans, grinding and screening, through an 80-mesh screen. The prepared enzyme is also satisfactory for this test.

(2) Sometimes the indicator is bleached during the incubation, in which case it is necessary to add another drop of indicator when the incubation is finished.

(3) This test depends upon the conversion of urea into ammonia by the ureolytic enzyme. The temperature of the incubation should not exceed 40°, since cyanamide may, on long standing, suffer a partial transformation into urea.

Estimation

Cyanamide.—Nearly all of the methods used for the determination of cyanamide depend upon the insolubility of its silver salt in ammoniacal solutions. This insolubility in a reagent which dissolves the silver salts of all of the related nitrogenous compounds, together with the characteristic colour of the silver precipitate, make the

accurate determination of cyanamide nitrogen a comparatively easy task. The estimation may be made by determination of the nitrogen content of the precipitated silver salts, by volumetric determination of the silver in the precipitate, or by titration of excess silver in the filtrate after the precipitation of the silver salts.

Methods depending upon determination of silver assume constancy of composition of the precipitate, an assumption not always realised. Although it can be shown that the ratio in the precipitated salts of silver to nitrogen is not entirely constant, it can also be shown that the precipitate contains all of the cyanamide nitrogen, and that no nitrogen compound other than cyanamide is present, except when the cyanamide occurs with very large amounts of dicyandiamide. Consequently, it has been found preferable to rely upon the determination of the nitrogen content of the silver salts, and to employ the less accurate silver titration only for control of routine operations where speed is a paramount consideration.

Determination of Cyanamide by the Kjeldahl Method.—Take a portion of the solution containing not more than 0.10 grm. of nitrogen, and dilute it to a volume of about 150 c.c., neutralise it with ammonia, and then add 5 c.c. of strong ammonia in excess. Now add a 5% silver nitrate solution, drop by drop and with constant stirring, until precipitation is complete and then add 5 c.c. in excess. Allow the liquid to stand for 10 minutes, with frequent stirring, and then pour it through a filter. Wash the precipitate with cold water until the washings are no longer alkaline to phenolphthalein. Place the funnel in the neck of a Kjeldahl flask, perforate the paper, and pour slowly over the paper 50 c.c. of 1:1 sulphuric acid in such a manner that the precipitate is completely dissolved. Wash the paper several times with hot water. Place the flask in a digestion rack and evaporate until fumes appear. Digest for 30 minutes, cool, dilute, make alkaline with caustic soda, and distil off ammonia after first adding sufficient sodium sulphide solution to precipitate silver salts completely. Absorb the ammonia in standard sulphuric or hydrochloric acid and back-titrate the excess.

Pinck (*Ind. Eng. Chem.*, 1925, **17**, 459) has shown that the silver cyanamide precipitate may occasionally carry down with it small amounts of dicyandiamide. Consequently, when the cyanamide occurs with large amounts of dicyandiamide, he recommends that

the original precipitate should be dissolved in dilute nitric acid, and the solution diluted to the original volume of precipitation. A few c.c. of ammoniacal silver are now added, followed by ammonia to slight alkalinity. The purified silver cyanamide may now be analysed by the Kjeldahl method or by titration as below.

Determination of Cyanamide by Silver Titration.—Precipitate the silver salts in ammoniacal solution as described above, filter and wash the precipitate. Perforate the filter paper, and pour slowly over the paper 50 c.c. of 1:4 nitric acid in such manner that the precipitate is completely dissolved. Dilute the mixture to 200 c.c., and titrate the silver with standard thiocyanate, using ferric nitrate solution as indicator.

If desired, the excess silver in the filtrate may be titrated instead of the silver in the silver salts. In that case make the precipitation with an excess of $N/10$ silver nitrate solution, filter, and titrate the excess of silver after making the solution acid with nitric acid. Obviously, this procedure is only applicable in absence of chloride or other compounds precipitated by silver in acid solution.

Xanthidrol Method.—The xanthidrol method of Fosse for the identification of urea was briefly described on page 592. Fosse has applied the same procedure to the determination of urea and also to the determination of cyanamide. For the latter determination the cyanamide is precipitated as silver cyanamide, which is then converted into urea and silver nitrate by treatment with nitric acid. The urea so produced is precipitated as xanthidrol and weighed. For details of the method the original should be consulted (*Compt. rend.*, 1924, 179, 214, 408).

Dicyandiamide.—Although the study of the analytical chemistry of dicyandiamide has been given much attention, no method for its determination has yet been developed which can be applied indiscriminately to all classes of mixtures. Owing to the disrepute of dicyandiamide in agricultural circles its estimation is frequently called for, and wholly erroneous results have frequently been reported by analysts not fully informed concerning the limitations of the analytical procedure relied upon. In the discussion of the analytical methods for dicyandiamide which follows we have described the methods which are most generally applicable and have attempted to point out the peculiar limitations of each method.

Methods Depending upon Precipitation of Silver Dicyandiamide.—The oldest method for the determination of dicyandiamide is due to Caro. The dicyandiamide solution, which may contain cyanamide, urea, ammonia or other nitrogenous compounds, is made ammoniacal and silver nitrate is added. Silver cyanamide is precipitated, whilst silver dicyandiamide, owing to its solubility in ammonia, remains in solution. The silver cyanamide is filtered off, caustic soda is added to the filtrate, and silver dicyandiamide is precipitated, together with the excess silver. The mixture is now boiled to remove ammonia, after which the silver precipitated is filtered off, washed, and its nitrogen content determined by the Kjeldahl method. The total nitrogen content of the silver precipitate is assumed to be dicyandiamide nitrogen.

It has been shown by later investigators that this early method gives accurate results only under exceptional circumstances. The prolonged boiling necessary completely to displace ammonia causes some decomposition of dicyandiamide, while urea is invariably carried down with the dicyandiamide precipitate. Since the two errors are of opposite sign, they may occasionally compensate each other; frequently, however, very misleading results are secured.

To avoid the prolonged boiling of the Caro method, Brioux divides the solution to be examined into two parts, precipitates cyanamide in one portion and cyanamide and dicyandiamide together in the other. Dicyandiamide is arrived at by difference. The Brioux method does not eliminate the very important error caused by inclusion of urea in the silver precipitate; in presence of much urea high values for the dicyandiamide content are inevitable, and there is no compensating error, as in the Caro method.

Hene and Van Haaren (*Z. angew. Chem.*, 1918, **31**, 129) have attempted to remedy this defect in the Brioux method by making a double precipitation of the silver dicyandiamide, hoping by so doing to eliminate urea from the precipitate. Their method represents a distinct improvement over the Brioux procedure, and although the precision is still not high, the method must occasionally be used for the examination of mixtures which do not lend themselves to analysis by more exact methods. A modified procedure following the suggestion of Hene and Van Haaren, which has been found to yield fairly reliable results, is as follows:

After determination of the cyanamide nitrogen by the Kjeldahl method as described on page 603, take another portion of solution, generally of the same size as the first, unless the sample is known to contain very large amounts of dicyandiamide, dilute to 250 c.c., add a volume of silver nitrate equal to that used for the cyanamide precipitation, and then a further amount depending upon the amount of dicyandiamide believed to be present. Now add slowly and with vigorous stirring 25 c.c. of 10% caustic soda solution. Stir for several minutes until the precipitate is coagulated and the supernatant liquid becomes clear, filter and wash the precipitate once with cold water. Wash the precipitate back into the original beaker with a small quantity of water, add nitric acid, drop by drop, until the precipitate is nearly all dissolved, dilute to the original volume, add 5 c.c. of silver nitrate and then 25 c.c. of the caustic soda solution, stirring as before. Filter and wash the precipitate twice with cold water. Transfer it to a digestion flask with 50 c.c. of 1:1 sulphuric acid and digest and distil as for cyanamide. The ammonia so recovered represents the cyanamide and the dicyandiamide. The dicyandiamide is then obtained by difference.

Precipitation as Silver Picrate Monocyanoguanidine.—The method is described by R. N. Harger (*J. Ind. Eng. Chem.*, 1920, **12**, 1107). It depends upon the fact that when a solution of silver picrate is added to a solution of dicyandiamide a double compound, $C_6H_2(NO_2)_3OAg.C_2H_4N_4$, silver picrate monocyanoguanidine, is formed. This compound crystallises in bright yellow platelets, softening at 235° . The method of analysis is as follows:—

To a 200 c.c. portion of solution add nitric acid, drop by drop, until the solution is acid to litmus. Now add 30 c.c. of a 5% silver nitrate solution and, if a precipitate of silver chloride is formed, as will usually happen when commercial materials are under examination, filter off the precipitate. Now add 200 c.c. of a saturated aqueous solution of picric acid, stir for a few minutes, leave in ice-water for 30 minutes, and filter on a Gooch filter. Wash first with water saturated with silver picrate monocyanoguanidine and then with ether, dry at 100° and weigh. To correct for the solubility of the precipitate, 0.0044 grm. should be added for each 100 c.c. of the solution from which the crystals were filtered.

The advantages of the Harger procedure over the silver precipitation method previously described are that it isolates the

dicyandiamide in such form that its identity can be established by observation, and that it is a direct, and not a difference, method. Notwithstanding these advantages, the Harger procedure contains numerous possibilities of error, and may, if applied indiscriminately, lead the analyst far astray.

The method assumes that no substance precipitable by picric acid alone is present. Unfortunately this is true of but few mixtures, since picric acid is far from being a specific precipitant. Harger himself has shown that considerable quantities of potassium salts interfere, and has stated that such interference can be recognised by the characteristic appearance of the potassium salt and by its solubility in the ether used for washing. It scarcely needs pointing out that there is grave danger of failure to identify potassium picrate by such uncertain tests. Harger has also called attention to the fact that certain organic extractives also yield precipitates with picric acid. This is so true that it may be said that few commercial fertiliser mixtures will fail to give some sort of precipitate with picric acid, particularly on standing.

Harger does not call attention to a still more serious defect of his procedure, *viz.*, that certain other cyanamide derivatives, guanylurea and guanidine for example, yield precipitates with picric acid which closely resemble the silver picrate dicyandiamide. If the method of Harger is to be applied to unknown mixtures, the analyst must therefore make sure that the precipitate which he obtains is formed by the combined action of silver and picric acid, and not by the picric acid alone. A duplicate sample must be treated with picric acid alone, the addition of the silver salt being omitted. If picric acid alone yields a precipitate, it is obviously unsafe to apply the Harger procedure.

Johnson (*J. Ind. Eng. Chem.*, 1921, **13**, 533) has described a volumetric method for the determination of dicyandiamide, which is somewhat similar to the Harger procedure. Dicyandiamide is precipitated as silver picrate dicyanoguanidine, and the amount of silver so consumed is a measure of the dicyandiamide content of the sample under examination.

By Conversion into Guanylurea.—By treatment with acid under properly controlled conditions dicyandiamide is quantitatively converted into guanylurea. The guanylurea may then be weighed, after precipitation as picrate or nickel salt. Analytical methods

depending upon this conversion are among the most reliable procedures available. They are particularly applicable when employed in connection with acetone extraction. Dicyandiamide is soluble in acetone, whilst guanylurea and biguanide, both of which yield an insoluble picrate and nickel salt, are insoluble. Application of the guanylurea conversion to the acetone extract of the material under examination affords one of the most accurate methods known for the dicyandiamide determination.

The original recommendation for this procedure is due to Von Dafert and Miklauz (*J. Soc. Chem. Ind.*, 1919, **38**, 837 A). Garby (*Ind. Eng. Chem.*, 1925, **17**, 266) has studied the method and recommends the following procedure: Evaporate to dryness a nitric acid solution of the sample under examination, thus hydrolysing the dicyandiamide to guanylurea, and precipitate the latter as nickel guanylurea. For the analysis of technical dicyandiamide, 0.10 grm. of the sample is treated with 20 c.c. of *N*/4 nitric acid, and the mixture is evaporated to dryness on a steam-bath. The guanylurea nitrate so formed is precipitated in the following way:

The reagents used are:—

(A) A 2% solution of ammonia saturated with nickel guanylurea prepared fresh every six months.

(B) 100 grm. mannitol made up to 1 litre with solution (A).

(C) 40 grm. of nickel nitrate and 20 grm. of ammonium nitrate dissolved in 100 c.c. of solution (A).

(D) 20% sodium hydroxide solution.

(E) Dilute ammonia solution.

The residue, after evaporation of the nitric acid solution, is taken up with 40 c.c. of solution (B), about 3 c.c. of solution (C) and sufficient of solution (D) are added, drop by drop, to produce a greenish-yellow colour in the solution. The solution turns green if dicyandiamide is absent, and greenish-yellow if it is present in large amounts. Precipitation is conveniently effected in a large stoppered weighing bottle, the solution being covered to prevent loss of ammonia, with consequent partial precipitation of nickel hydroxide. After standing over night the green-coloured solution is filtered through a weighed Gooch crucible and washed with 100 c.c. of solution (E). After drying at 125° for one hour the precipitate is weighed as $\text{Ni}(\text{C}_2\text{N}_4\text{H}_6\text{O})_2$. The factor for conversion to dicyandiamide is 0.645.

In the event of the samples to be examined being solutions containing guanylurea and biguanide, 25 c.c. of the solution, containing 0.1 to 0.4 grm. of dicyandiamide, are placed in a 50 c.c. calibrated flask and treated with 3 c.c. of solution (B), 2.5 to 3 c.c. of concentrated ammonia, 1 to 3 c.c. of solution (C), and sufficient of solution (D) to produce the colour changes described above. The solution is set aside in the stoppered flask for 2 to 3 hours, then made up to volume and filtered, a 25 c.c. aliquot portion acidified to $N/4$ with nitric acid, and the dicyandiamide determined after evaporation to dryness as described above.

The interference of guanylurea and biguanide is conveniently avoided, when solids are under examination, by extracting the sample in a Soxhlet extraction apparatus for two or three hours with acetone. The acetone extract is evaporated to dryness, and the residue treated with nitric acid. Guanylurea and biguanide salts are not soluble in acetone and are thus eliminated.

Instead of using the nickel salt, the picrate of guanylurea may be precipitated after the evaporation with nitric acid, provided, of course, guanylurea and biguanide have first been eliminated or determined. The details of the precipitation of guanylurea with picric acid are given in the section on the determination of guanylurea.

Attention should be called to the fact that the hydrolysis of dicyandiamide must be carried out under defined conditions, since strong acids may decompose the guanylurea salts, to yield guanidine salts. As an alternative to the evaporation to dryness with $N/4$ nitric acid, the writer has found that quantitative conversion to guanylurea is effected by heating 50 c.c. of solution with 15 c.c. of concentrated hydrochloric acid for 15 minutes at the temperature of a boiling water bath. Prolonged heating results in the production of guanidine.

Urea.—The earlier procedure for the determination of urea in cyanamide mixtures involved determination of total nitrogen and subtraction from this value of the combined cyanamide and dicyandiamide nitrogen. Obviously, this method disregarded the possibility of the presence of nitrogenous forms other than the three just mentioned. Of the methods used for the determination of urea in physiological chemistry, for example the measurement of the nitrogen evolved by treatment with sodium hypobromite, or pre-

precipitation with mercurous nitrate, none was applicable in presence of cyanamide. Fox and Geldard (*J. Ind. Eng. Chem.*, 1923, **15**, 743) have made a study of the analytical chemistry of urea to determine the method most applicable to this important determination in cyanamide mixtures, and their conclusion is to the effect that the urease method of Marshall is the only satisfactory method. With this conclusion the writer agrees, and it has been considered unnecessary to present the details of any other method.

The urease method is based upon the fact that in approximately neutral solution urea is quantitatively converted into ammonium carbonate by the enzyme urease which occurs principally in the soya or jack bean. Fox and Geldard recommend the following procedure for the preparation of the enzyme: A few grm. of jack bean flour are extracted with 20 times its weight of water for 10 to 15 minutes, exactly neutralised with hydrochloric acid (1 c.c. of 0.1*N* HCl to 1 grm. of jack bean flour), and filtered. 10 c.c. of this extract are sufficient to convert 0.1 grm. of urea into ammonia in less than one hour.

For the urea determination weigh out 0.5 grm. of the sample and dissolve it in 250 c.c. of water. Pipette 25 c.c. into a tall form wide-mouthed bottle of about 100 c.c. capacity, add a few drops of methyl red indicator, and bring to exact neutrality, using 0.1*N* NaOH. Add 10 c.c. of neutral urease and leave for 1 hour, keeping the mouth of the bottle closed. Add from a burette a measured excess of 0.1*N* HCl; insert in the bottle a glass tube, with a bulb and fine holes at its lower end, and rapidly aerate with purified air for 5 to 10 minutes to remove carbon dioxide. Two or three drops of caprylic alcohol or liquid petrolatum added to the solution will prevent frothing. After aerating titrate to exact neutrality with 0.1*N* NaOH.

So far as is known, the urease method is specific for urea. Cyanamide, dicyandiamide and guanylurea have no influence. In presence of calcium salts the calcium must be removed by use of sodium carbonate, and the excess of carbonate destroyed by acidification and aeration before adding the urease. In the analysis of fertiliser mixtures containing soluble phosphates the phosphate must be removed with barium hydroxide, and the excess of barium with sodium carbonate. The excess of sodium carbonate is then removed by acidification.

The writer has used with success the prepared urease enzyme, which may now be obtained from the chemical supply houses. This obviates the somewhat troublesome preparation of the bean flour.

The xanthidrol method of Fosse has been mentioned on page 592. Johnson (*J. Ind. Eng. Chem.*, 1921, 13, 535) describes a method for the determination of urea based on the formation of a difficultly soluble salt with oxalic acid. By selecting the proper conditions the solubility of the urea oxalate can be made so slight as to permit a quantitative determination. For details the reader is referred to the original.

Guanylurea.—The nickel guanylurea method for the determination of guanylurea has been described in the section on the determination of dicyandiamide. Guanylurea is also conveniently determined as the picrate, the method being applicable, of course, only in absence of other substances precipitated by picric acid. A procedure recommended by the Fixed Nitrogen Research Laboratory is as follows:—

A sample is taken containing approximately 0.1 gram. of guanylurea sulphate in exactly 25 c.c. of a neutral solution. To this is added 5 c.c. of a clear solution of picric acid in 95% alcohol containing 6 gram. of picric acid to 100 c.c. of solution. This furnishes about 100% excess of picric acid. A yellow precipitate of guanylurea picrate forms quite rapidly. The solution should be stirred for several minutes and then allowed to settle until the supernatant liquid is clear. The liquid is decanted off and sucked through an alundum filter crucible that has been previously dried and weighed. The precipitate is washed twice, each time with 5 c.c. of cold water, and as much of the precipitate as possible transferred to the crucible. The precipitate still remaining in the beaker is then washed into the crucible by a stream from a wash-bottle, about 5 c.c. of water again being used. The precipitate is dried at 110° for one hour, cooled and weighed.

A correction factor is to be added to the weight of the precipitate to account for the small amount of picrate remaining in solution. This amounts to 0.0044 gram. of picrate when the quantities of liquid specified above are used. This factor depends entirely upon the amount of solution from which the material is precipitated, the amount of alcoholic picric acid solution added, and the quantity of wash-water used. It is therefore necessary to follow closely the

directions given. However, if the quantities of all liquids used are doubled, correct results may be obtained by doubling the correction factor.

The molecular weight of guanylurea picrate is 331, $(C_6H_2(NO_2)_3-OH.C_2H_6N_4O)$.

Guanidine.—All described methods for the determination of guanidine depend upon precipitation and weighing of the picrate. Vozarik (*Z. angew. Chem.*, 1902, **15**, 670) gives the following directions for the analysis of guanidine by the picrate method: 8 gm. of the guanidine salt are dissolved in water and a little ammonia, and the solution is diluted to 1 litre. When clear, 25 c.c. are mixed with 100 c.c. of ammonium picrate solution, prepared by dissolving 8 gm. of ammonium picrate, 0.075 gm. of guanidine picrate, and 5 c.c. of ammonia (sp. gr. 0.9) in a litre of water. After 6 to 12 hours the precipitate is collected on a Gooch crucible and washed with picrate solution. After the adhering liquid has been removed by suction, as far as possible, the crucible and contents are dried at 110° and weighed. The precipitate retains 1%, and the asbestos layer 2.5% of its weight of ammonium picrate, for which allowance must be made. One mol. of the picrate is equivalent to 1 mol. of guanidine.

Instead of using the procedure of Vozarik we may determine guanidine by precipitating and weighing the picrate, following exactly the procedure just described for the precipitation and weighing of guanylurea picrate. The solubility correction to be applied in this case is 0.015 gm. of picrate for each 25 c.c. of solution.

Ammonia.—Some ammoniacal nitrogen is always formed when calcium cyanamide is added to, and allowed to react in, the soil or in chemical mixtures. Frequently also, particularly in the case of fertiliser mixtures, the calcium cyanamide is purposely mixed with ammonium salts. The analyst is therefore frequently confronted with the necessity of determining ammonium salts in mixtures in which cyanamide or some of its reaction products is present.

Magnesia Method of A. O. A. C.—The official method of the Association of Official Agricultural Chemists for the determination of ammoniacal nitrogen in fertiliser and similar mixtures is as follows: Place 0.7 to 3.5 gm., according to the ammonia content of the substance to be analysed, in a distilling flask with about 200 c.c. of water, and add 5 gm. or more of magnesium oxide free from car-

bonate. Then connect the flask with a condenser and distil 100 c.c. of the liquid into a measured quantity of standard acid and titrate with standard alkali solution, using cochineal or methyl red indicator.

This method gives good results when inorganic mixtures are under examination, but becomes somewhat less accurate when organic ammoniates, particularly cyanamide, are present.

Folin's Method.—Better results may be secured by application of the aeration method, as developed by Folin for the analysis of urine. The procedure recommended for fertilisers is as follows (Folin and Bosworth, *J. Ind. Eng. Chem.*, 1913, 5, 485):—Two grm. of fertiliser are placed in a 100 c.c. graduated flask, about 50 c.c. of water added, and then 25 c.c. of approximately N/hydrochloric acid. The volume is made up to 100 c.c. with water, the contents shaken, allowed to stand for a few minutes, and shaken again. The flask is allowed to stand until the heaviest of the undissolved particles have settled. 5 c.c. of the supernatant liquid are transferred by means of a pipette to the tube of a Folin bubbling apparatus, and 2 c.c. of a saturated solution of potassium oxalate, a few drops of kerosene, and finally 2 c.c. of a saturated solution of potassium carbonate are added. The Folin apparatus, which is described in detail in the *J. Biol. Chem.*, (1912, 11, 493), may be obtained from most dealers in chemical glassware.

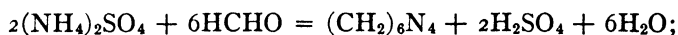
After addition of the potassium carbonate the apparatus is immediately closed, and air is passed through for 10 to 20 minutes. The ammonia liberated is collected in a flask containing 20 c.c. of N/70 HCl. After the distillation the acid is titrated back with N/70, using alizarin red as indicator.

Formol Titration.—The formol titration is based upon the fact that ammonium salts react readily with formaldehyde, yielding hexamethylenetetramine and liberating the free acid, which may then be titrated. The following procedure is recommended:—Place from 25 to 100 c.c. of the solution containing not more than 0.25 grm. of ammonium sulphate; in a 150 c.c. beaker, add 1 drop of methyl red indicator, and then make just acid with hydrochloric acid. If carbonates are present, aerate the solution vigorously to remove carbon dioxide. Now make exactly neutral with N/10 NaOH, add 10 c.c. of 40% formalin solution which has previously been neutralised to phenolphthalein indicator, and then add N/10 NaOH until a slight excess is present. Allow the mixture to stand for 30

minutes, and titrate the excess sodium hydroxide with $N/10$ sulphuric acid.

This titration is not interfered with by cyanamide, dicyandiamide or urea. Guanylurea salts react with formaldehyde in the same manner as ammonium salts, the acid of the salt being liberated. If present, guanylurea must be determined separately and suitable allowance made, bearing in mind that one mol. of guanylurea is equivalent to one mol. of ammonia.

The reaction in the case of the ammonia is—



each mol. of ammonia sets free by reaction with formaldehyde one equivalent of hydrogen ion, which can be titrated with NaOH .

If carbonates are present, they must be removed by aeration, as indicated above. Phosphates also interfere. The procedure for the analysis of commercial fertiliser mixtures, in which soluble phosphates are nearly always present, is described on page 615. The formol titration is not applicable to mixtures containing materials formed by the acid hydrolysis of proteins (ammoniated base), owing to reaction between formaldehyde and the amino acids.

Analysis of Commercial Calcium Cyanamide

Estimation of Total Nitrogen.—The Gunning method is used by the American Cyanamid Company for the determination of the total nitrogen content of calcium cyanamide (Bacon, *Amer. Fertilizer*, 1922, 56, 55).

Weigh out 0.7 grm. of the finely-ground sample and transfer it to a dry Kjeldahl flask. Add 5 grm. of powdered potassium sulphate and mix thoroughly. Run in 35 c.c. of concentrated sulphuric acid, heat for a few minutes over a small flame, and then over a full Bunsen flame for 0.5 to 1.5 hour. Cool, dilute, and distil 250 c.c. into a measured volume of $N/2$ sulphuric acid after addition of excess of caustic soda. Titrate the excess acid with $N/10$ alkali, using methyl red as indicator.

Estimation of Cyanamide Nitrogen.—Weigh out 2.5 grm. of the calcium cyanamide into a 500 c.c. shaking flask, and quickly add about 300 c.c. of ice-water. Shake vigorously for several minutes

to break up all lumps. Add a piece of litmus paper, and then 1:1 nitric acid, drop by drop and with vigorous stirring, until the mixture remains just acid to litmus. Stopper the flask and shake, preferably mechanically, for one hour. Make up to volume, shake, allow the insoluble matter to settle, and pour through a dry filter. Take a 100 c.c. portion of the clear filtrate, make ammoniacal and add silver nitrate, as described on page 602.

Estimation of Dicyandiamide Nitrogen.—When the silver precipitation methods (described on page 604) are to be employed another 100 c.c. portion of solution prepared as for the cyanamide analysis above is taken.

When the analysis is to be made by the method of Harger (described on page 605) 5 to 10 gram. of the substance are extracted by shaking for 30 minutes with 300 c.c. of water, and dilute nitric acid is then added until the mixture is acid to litmus, and then 75 c.c. of 5% silver nitrate solution are added. The mixture is made up to 500 c.c. and filtered. For analysis a 100 or 200 c.c. aliquot part is taken, and aqueous picric acid is added as described.

When the dicyandiamide is to be estimated by conversion into guanylurea Garby (*loc. cit.*) recommends that a sample of such size as to give an aliquot part containing 0.05 to 0.2 gram. of dicyandiamide should be extracted with acetone in a shaking machine for 2 or 3 hours, the solution filtered, and an aliquot part taken to dryness on a steam-bath. If the material contains oil, the acetone-soluble residue is extracted with absolute ether, and the residue treated with nitric acid and again evaporated to dryness, as described on page 607.

Estimation of Urea Nitrogen.—Fox and Geldard (*loc. cit.*) recommend the following procedure for the analysis of hydrated and oiled cyanamide:—Extract 2 grms. of calcium cyanamide with 400 c.c. of water for 2 hours; add 2 gram. of anhydrous sodium carbonate to precipitate the calcium, and continue to shake for another half hour. Filter the extracts through a dry filter, and take 25 c.c. aliquot parts for urea analysis. Pipette the aliquot part into bottles, make the solution distinctly acid with dilute hydrochloric acid, and aerate until all the carbon dioxide is expelled, as shown by the sharpness of the end-point in the subsequent neutralisation. Then make the solution exactly neutral, using methyl red indicator, and determine urea as described on page 608.

Analysis of Phosphatic Fertiliser Mixtures

A few special analytical problems are presented by mixed fertilisers in the compounding of which calcium cyanamide has been employed. Unchanged cyanamide is rarely found in such mixtures, except for a very brief time after compounding. Consequently, the analyst need not be surprised at failure to detect cyanamide in mixtures prepared with calcium cyanamide additions. Cyanamide, if present, may be detected and determined by the methods already described, no special procedure being necessitated by presence of soluble phosphates.

For determination of dicyandiamide in such mixtures an acetone extraction is recommended, dicyandiamide being determined in the extract after conversion to guanylurea. When the Harger method is to be applied, the following modification is to be adopted:—Stir 20 grm. of the sample with 50 c.c. of water, next add 100 c.c. of a saturated solution of barium nitrate, and then barium hydroxide until the mixture reacts alkaline to litmus. Make up to 500 c.c. and take a 200 c.c. aliquot portion for analysis. In presence of organic materials which produce gelatinous precipitates with picric acid, cotton seed meal for example, lead acetate solution should be added after the addition of the barium hydroxide, to precipitate the interfering compounds.

For the determination of urea, extract an 8 grm. sample with 400 c.c. of water, precipitate the phosphate completely with barium hydroxide, and remove the excess of barium with a few grm. of sodium carbonate. Filter through a dry filter and take aliquot portions for the urea determination, as described in the preceding section on the analysis of commercial calcium cyanamide.

For guanylurea, grind a 4 grm. sample in a mortar with 25 c.c. of a thick suspension of milk-of-lime. Pour the mixture on to a filter paper and wash with water in small portions until the filtrate and washings are exactly 100 c.c. To 25 c.c. portions of the filtrate, representing one gram of sample, apply the picric acid or the nickel precipitant as described on pages 607 and 610.

For the determination of ammonium salts the magnesia distillation or the aeration method of Folin may be applied directly. If the formol titration is to be employed, soluble phosphates must be removed by the following procedure:—Weigh into a 400 c.c. beaker a sample

containing not over 3 grm. of P_2O_5 . Add 150 c.c. of a saturated solution of barium nitrate and stir for ten minutes. Place the beaker in an ice-bath, and add from a burette a saturated solution of barium hydroxide, continuing the addition until the mixture is alkaline to litmus, and then adding 2 c.c. in excess. Test for complete precipitation of phosphate with a little more barium nitrate solution. Filter and wash the precipitate, making up the filtrate and washings to 500 c.c. Place 25 c.c. of the solution so prepared in a beaker, add 1 drop of methyl red indicator, and make just acid with hydrochloric acid. Continue the analysis as described on page 612.

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THE PROTEINS

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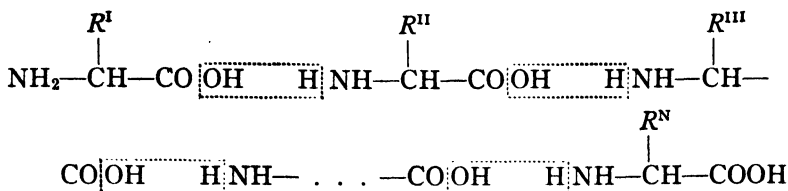
Under the generic name of proteins is classed a large number of highly complex nitrogenous substances which form a considerable part of the solid constituents of blood, muscles, glands, and other organs of animals, and which occur in smaller quantities in almost every part of plants.

The typical member of the class is albumin, the chief protein constituent of the white of egg, and at one time the term "albumin" was employed to designate all other members of the group. It was decided, however, by a joint committee of the Physiological and Chemical Societies of London, and by a committee of the American Society of Biological Chemists to use the generic name of "protein." The same committee also recommended a general method of classification of the proteins which is adopted in the following pages.¹

Our knowledge of the constitution and structure of the proteins is based largely on the researches of Emil Fischer and his pupils, carried out in the early years of the present century, and collected together in the form of a monograph. (E. Fischer, "*Untersuchungen über Aminosäuren, Polypeptide und Proteine*," Berlin, 1906) Fischer elucidated many points of structure, and succeeded in preparing synthetically a number of substances of protein-like character, although even now no naturally-occurring protein has been synthesised.

From the researches of Fischer, it would seem that the proteins belong to the class of substances designated as "polypeptides," which are formed by the condensation of amino-acids according to the scheme:—

¹ The classifications adopted by the English and American societies differed slightly from one another. That suggested by the Americans contained a larger number of classes and was rather more complete. With one or two minor alterations, it has been adopted therefore, in preference to the English classification.

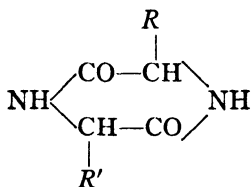


R in the above formula may represent either a simple alkyl or aryl radicle, or a similar group in which a hydrogen atom is substituted by either an amino group or a carboxyl group.

The most complex polypeptide prepared by Fischer contained eighteen amino-acid residues; Abderhalden has prepared a compound containing nineteen such residues. These compounds possess many of the properties of the natural proteins.

Further, on hydrolysis of proteins, either by acids or by the action of certain enzymes, mixtures of peptides of varying complexity are obtained, which are themselves hydrolysable to simple amino-acids.

During recent years, the theory which regards the proteins purely as complex polypeptides has been questioned by some workers, more particularly by Abderhalden and his school. Abderhalden has arrived at the conclusion that the essential structure of the protein molecule involves the presence of the diketopiperazine ring (the amino-acid anhydride structure):—



Abderhalden has succeeded in isolating such substances from among the products of protein degradation, and has sought to prove that they were not formed from peptides or amino-acids by secondary reactions. His experiments and conclusions have been the subject of much criticism, and at the moment the most that can be said is that while certain proteins may contain a part of their nitrogen in the form of such diketopiperazine rings, there is no reason to doubt that the polypeptide structure put forward by Fischer is a more correct representation of the constitution of most proteins.

The proteins differ from one another very markedly in their physical properties and they also differ from one another chemically, in that they yield varying amounts and varying numbers of amino-acids on hydrolysis. The original conception of Mulder, to whom the name "protein" is due, that only one such substance exists, which forms the basis of all the albuminoid substances, which are said to differ from one another only in physical properties, is therefore incorrect. By the elaboration of methods for approximately quantitative separation of the hydrolysis products of the proteins, the way was indicated for distinguishing with some degree of certainty the individual proteins.

The following amino-acids have been isolated as protein hydrolysis products:—

HYDROLYSIS PRODUCTS OF PROTEINS

A. Monoamino-monocarboxylic acids

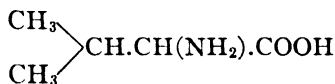
1. Glycine, $C_2H_5O_2N$, or aminoacetic acid.



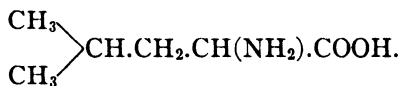
2. Alanine, $C_3H_7O_2N$, or α -aminopropionic acid.



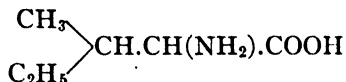
3. Valine, $C_5H_{11}O_2N$, or α -aminoisovaleric acid.



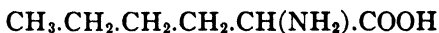
4. Leucine, $C_6H_{13}O_2N$, or α -aminoisocaproic acid.



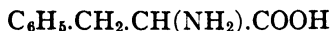
5. Isoleucine, $C_6H_{13}O_2N$, or α -amino- β -ethyl- β -methylpropionic acid.



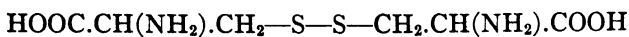
6. Caprine, $C_6H_{13}O_2N$, or α -aminocaproic acid.



7. Phenylalanine, $C_9H_{11}O_2N$, or β -phenyl- α -aminopropionic acid



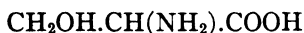
8. Cystine, or dicysteine, $C_6H_{12}O_4N_2S_2$, or di(β -thio- α -aminopropionic acid).



9. Methionine, $C_5H_{11}O_2NS$, or γ -methylthiol- α -aminobutyric acid.



10. Serine, $C_3H_7O_3N$, or β -hydroxy- α -aminopropionic acid.



11. Hydroxyaminobutyric acid, $C_4H_9O_3N$

12. Hydroxyvaline, $C_5H_{11}O_3N$

13. Tyrosine, $C_9H_{11}O_3N$, or p- β -hydroxyphenyl- α -aminopropionic acid.



B. Monoamino-dicarboxylic acids

14. Aspartic acid, $C_4H_7O_4N$, aminosuccinic acid.



15. Glutamic acid, $C_5H_9O_4N$, α -aminoglutaric acid.

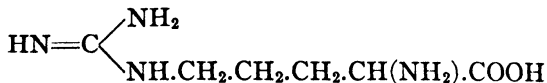


16. β -hydroxyglutamic acid, $C_5H_9O_5N$.



C. Diamino-monocarboxylic acids

17. Arginine, $C_6H_{14}O_2N_4$ or α -amino- δ -guanidinevaleric acid.



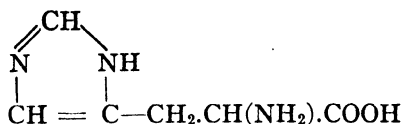
18. Lysine, $C_6H_{14}O_2N_2$, or $\alpha\epsilon$ -diaminocaproic acid.



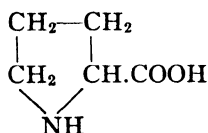
19. Hydroxylysine, $C_6H_{14}O_3N_2$, or hydroxy- $\alpha\epsilon$ -diaminocaproic acid.

D. Heterocyclic compounds

20. Histidine, $C_6H_9O_2N_3$, or β -iminazole- α -aminopropionic acid.

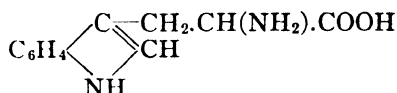


21. Proline, $C_5H_9O_2N$, or α -pyrrolidinecarboxylic acid.



22. Hydroxyproline, $C_5H_9O_3N$.

23. Tryptophane, $C_{11}H_{12}O_2N_2$, or β -indole- α -aminopropionic acid.



24. Protocline, $C_8H_{15}O_3N_3$.

Several of the above substances occur in optically active and inactive forms.

PERCENTAGES OF AMINO-ACIDS DERIVED BY HYDROLYSIS FROM CERTAIN TYPICAL ANIMAL PROTEINS

	Salmine ¹	Egg-albumin ²	Serum ³ albumin	Serum ⁴ globulin	Caseinogen ⁵	Gelatin ⁶
Glycine.....			0	3.25	0.45	25.5
Alanine.....		2.1	2.7	2.2	1.85	8.7
Valine.....	4.3			+	7.9	0
Leucine.....		6.1	20.5	18.7	9.7	7.1
Phenylalanine.....		4.4	3.1	3.8	3.9	1.4
Tyrosine.....		1.1	2.1	2.5	4.5	0
Serine.....	7.8		0.6		0.5	0.4
Cystine.....		0.3	4.2	1.5		0
Proline.....	11.0	2.3	1.0		7.6	9.5
Hydroxyproline.....					+	14.1
Aspartic acid.....		1.5	3.1	2.5	1.8	3.4
Glutamic acid.....		8.0	7.7	8.5	21.8	5.8
Arginine.....	87.4		4.9	3.9	3.8	8.2
Lysine.....	0		13.2	8.9	7.6	5.9
Histidine.....	0		3.4	2.8	2.5	0.9
Tryptophane.....		+	+	+	(1.5)	0

¹ Kossel and Dakin, *Z. physiol. Chem.*, 1904, **41**, 407.

² Abderhalden and Pregl, *Z. physiol. Chem.*, 1905, **46**, 24; Möerner, *ibid.*, 1901, **34**, 207.

³ Abderhalden, *Z. physiol. Chem.*, 1903, **37**, 495.

⁴ Abderhalden, *Z. physiol. Chem.*, 1905, **44**, 17.

⁵ Foreman, *Biochem. J.*, 1919, **13**, 378.

⁶ Dakin, *J. Biol. Chem.*, 1920, **44**, 499.

The Hydrolysis of the Proteins and the Separation of the Amino-acids

As stated above, the proteins differ from one another in the number and quantities of the amino-acids which they yield on hydrolysis. The quantitative estimation of the hydrolysis products is, therefore, an important operation when it is necessary to establish the identity or difference of proteins of various origins. Unfortunately, the operation of separating the various amino-acids, especially the mono-amino-acids, is for the most part a very complex and difficult one, requiring relatively large amounts of materials, and yielding, at any rate in the case of the mono-amino-acids, barely approximately quantitative results. It cannot therefore be included among the ordinary routine methods for the analytical investigations of the proteins. Certain individual amino-acids, such as tyrosine and ammonia, can, however, be estimated fairly readily, and certain groups, such as the mono-amino- and diamino-acids can also be easily estimated. The processes employed in these estimations will be described below, when dealing with the routine analytical methods. As, however, the estimation of the amino-acids is a process of such fundamental importance for the general chemistry of the proteins, and is continually forming the subject of new investigations, which may finally result in simplification and greater accuracy, if not with all, at any rate with certain of the amino-acids, it has been thought advisable to give a somewhat detailed account of the methods although they can only be applied when large amounts of material are available, and a somewhat lengthy research is possible.

A. THE SEPARATION OF THE MONO-AMINO-ACIDS

The processes originally devised by Fischer for the separation of the mono-amino-acids from among the hydrolysis products of proteins consisted in the esterification of the amino-acids, and the separation of the esters into fractions by distillation under reduced pressure, the esters of the individual amino-acids having more or less distinct boiling points. Within the last ten years, modifications of the ester method have been introduced, as well as methods designed to obviate the use of the esterification process.

In all methods the protein is first hydrolysed by means of 25% sulphuric or 25% hydrochloric acid, (hydrofluoric acid has also been employed). It has been shown (Knaggs, *Biochem. J.*, 1923, 17,

barium salts of aspartic acid, glutamic acid, and hydroxyglutamic acid separate as a crystalline mass; these are removed, washed with cold dilute alcohol, and may be decomposed and the individual amino-acids separated as described below (p. 637). After complete removal of the alcohol and barium from the filtrate, the basic amino-acids are removed by precipitation with phosphotungstic acid in the presence of sulphuric acid. Nitrogen is estimated in the liquid, and the concentration adjusted so that the liquid contains 2% of N. Sufficient sulphuric acid is added to bring the concentration to 5% H_2SO_4 , and phosphotungstic acid reagent (50 gm. pure phosphotungstic acid¹ in 1,000 c.c. of 5% sulphuric acid) is added in the cold so long as a heavy precipitate forms. After standing for 24 hours the precipitated phosphotungstates are removed and washed with 5% sulphuric acid containing a little phosphotungstic acid, or with dilute hydrochloric acid; the further treatment of this fraction is described in detail below (p. 638).

The filtrate, after quantitative removal of the phosphotungstic and sulphuric acid by means of barium hydroxide, contains the monoamino-acids. After evaporation of the filtrate to a suitable concentration, these may be separated by one of the methods outlined below.

I. Methods Involving Esterification of the Amino-acids

In the method originally used by Fischer, the solution of the amino-acids is concentrated to a syrup, and this is dissolved in about three times its weight of absolute alcohol. The amino-acids are then converted into their esters by saturating the mixture with dry hydrochloric acid gas, and after allowing the mixture to stand overnight, evaporating it down under diminished pressure on a water bath. The residue is then redissolved in the same quantity of absolute alcohol, the solution is again saturated with hydrogen chloride and again evaporated under diminished pressure. The process is, if necessary, repeated a third time. The acids are thus converted into the *hydrochlorides of the esters*. A modification of this process, which in the hands of Osborne and his co-workers has yielded satisfactory results, has been suggested by Phelps and Tellotson. (*Amer. J. Sci.*, 1907, **24**, 194). This consists in treating the syrup with alcoholic hydrogen chloride and zinc chloride, and

¹ It is essential that a reliable preparation of phosphotungstic acid be used.

distilling through it, when heated to 110° , alcohol containing 10% of anhydrous hydrogen chloride gas (see also Osborne and Breese Jones, *Amer. J. Physiol.*, 1910, **26**, 212). When *glycine* is present in appreciable quantities, the greater part of the hydrochloride of its ester separates out, when the concentrated solutions of the hydrochlorides of the esters are allowed to stand at 0° for 24 hours, and can be filtered off at this low temperature in a nearly pure state.

The esters must then be set free from their hydrochlorides. This is a process requiring care and experience to be carried out in a satisfactory manner. The original process of Emil Fischer consists in adding 33% sodium hydroxide to the syrup dissolved in an equal volume of water, covered with twice the volume of cold ether, and cooled to 0° , until the excess of free acid is neutralised, and then adding gradually an excess of granulated potassium carbonate with continual shaking, until a pasty mass is formed. During the operation, the supernatant ether is several times poured off and renewed. Some of the esters are so readily hydrolysed by acid that even with great care the conversion of the hydrochlorides into free esters is not quantitative. For this reason, the residue left after treating with alkali and ether should be again treated with alcohol and hydrogen chloride; and the greater part of the inorganic salt which separates should be filtered off. The hydrochlorides of the esters thus obtained should be again treated in the same way as before. The process is repeated a third or fourth time, if necessary. Large quantities of ethereal extracts of the esters are thereby obtained, which are dried for about 15 minutes with potassium carbonate, and then for twenty-four hours with fused sodium sulphate. The residue, after distillation of the ether in a water-bath, is then ready for fractionation. It should be noted that some of the lower boiling esters (*glycine* and *alanine*) distil over with the ether. They can be recovered in the form of the hydrochlorides by extracting the ethereal distillate with hydrochloric acid. The fraction thus obtained can be treated in a similar way to the lowest boiling fraction got by distillation.

Various modifications of the above process have been suggested, of which the most useful is probably that due to Foreman (*Biochem. J.*, 1919, **13**, 378), in which the amino-acids are converted into their lead salts before esterification. The amino-acid solution used should

have a concentration of about 10%-15%; freshly precipitated lead hydroxide (about one-third the weight of the amino-acids present) is added, and the solution is heated for 30 minutes by passing steam into it; the liquid is then filtered, a small amount of humin material being removed. Litharge (4 or 5 times as much as the amount of lead hydroxide used) is now added, and the mixture further heated by steam for one hour; by this time the amino-acids are completely in the form of their soluble lead salts. The liquid is filtered, and the filtrate is evaporated completely to dryness, after which the solid residue is powdered, and well dried in an oven at 100°.

The lead salts are now suspended in absolute alcohol, and dry hydrogen chloride is passed in until the liquid is saturated with the gas; after cooling to 0°, a further amount of dry hydrogen chloride is passed. The lead chloride formed is filtered off, while the amino-acids remain in the alcoholic solution in the form of their ester hydrochlorides. From the filtrate most of the hydrochloric acid is removed by evaporation *in vacuo*; ammonia (in alcoholic solution) is added until the liquid is only faintly acid, when the precipitated ammonium chloride is filtered off. The alcohol is removed as completely as possible from the solution by evaporation, and the ester hydrochlorides are dissolved from the resulting syrup by dry chloroform; a small residue of ammonium chloride remains here. Sufficient anhydrous barium oxide is added to the chloroform solution to decompose the ester hydrochlorides; this is added in small amounts, the liquid being kept cool and well shaken. The barium residues are washed with dry chloroform; the chloroform is evaporated from the liquid and washings, and the residual syrup is taken up in anhydrous ether. (Some nitrogen remains in the barium residues; these are therefore decomposed with sulphuric acid, and from the extract, the lead salts are prepared and esterified as before.) The subsequent treatment of the esters follows the Fischer method.

✓The advantages of the method of Foreman lie in the fact that esterification of the lead salts is complete, and that there is minimum contact with alkali and water, and therefore less loss of esters by hydrolysis during the manipulations. The yield of esters is, in fact, almost quantitative.

More recently the use of butyl esters rather than the ethyl esters has been suggested; it is said that these are more easily and completely fractionated.

Fractionation of the Esters of the Amino-acids, and Examination of the Individual Fractions.—The esters prepared by the above-described methods are obtained in the form of a dark brown oil, which is then subjected to fractional distillation. The lower fractions are distilled off under a pressure of 10–12 mm., which can be obtained by means of an ordinary water pump; the higher fractions, on the other hand, require a lower pressure, which can best be obtained with the use of a mechanically driven double Geryk pump, between which and the distillation apparatus is interposed a condensing tube cooled with liquid air. In the absence of such a tube, the vapours due to the slight decomposition taking place during distillation are apt to raise the pressure and contaminate the pump. Levene and Van Slyke (*Biochem. Z.*, 1908, 10, 214) have substituted for the liquid-air cooled condenser a wash-bottle containing sulphuric acid and cooled by a freezing mixture. Another method suggested for obtaining a low vacuum consists in the employment of coconut charcoal cooled in liquid air. The fraction collected will depend on the amino-acids obtained, but the following may be separated in a typical hydrolysis. *Fraction I.*—Below 40° (vapour) under 10 mm. pressure, (esters of glycine and alanine). *Fraction II.*—40–60° under 10 mm. (esters of alanine, leucine and proline). *Fraction III.*—60–90° under 10 mm. (esters of valine, leucine and proline). *Fraction IV.*—100° (water bath) under 0.5 mm. pressure (esters of leucine and proline). *Fraction V.*—100–130° (oil-bath) under 0.5 mm. pressure (esters of phenylalanine, aspartic acid, glutamic acid and serine). *Fraction VI.*—130–160° (oil-bath) under 0.5 mm. pressure (esters of phenylalanine, aspartic acid, glutamic acid and serine).

The more recently discovered hydroxyamino-acids were not isolated by the ester method, and it is not certain in which distillation fraction they would appear. Judging from analogy with serine, it is likely that they would be found in Fractions V and VI, and in the distillation residue.

Each fraction is then hydrolysed separately, and the constituents as far as possible quantitatively separated. The lower fractions are hydrolysed by simply boiling with 5 to 6 times their volume of water until the alkaline reaction disappears, whereas the higher fractions, which contain the diamino-acids, must be hydrolysed with barium hydroxide solution, as hydrolysis with water alone is ineffective.

A valuable critical examination of the sources of error involved in the quantitative examination of the hydrolysis products by the ester method has been made by T. B. Osborne and Breese Jones (*Amer. J. Physiol.*, 1910, **26**, 305). They point out that the chief sources of error are due to the following factors: (a) incomplete hydrolysis; (b) incomplete esterification; (c) loss in distillation, (i) in the distillation of the ether, and (ii) in destructive secondary decomposition; (d) loss in separation of the individual acids from the various fractions.

II. Methods Not Involving Esterification of the Amino-acids

Of recent years methods have been introduced with the object of avoiding, in greater or less degree, the use of the esters; such, for instance, is the method of separating the mono-aminoacids by making use of the varying solubilities of certain of their metallic salts, and of the amino-acids themselves.

A method, was introduced by Dakin (*Biochem. J.* 1918, **12**, 290) whereby the amino-acids could be obtained solid, in a form convenient for further separation either by means of salts or by the ester method. The process consists in the prolonged extraction of an aqueous solution of the amino-acids by means of butyl alcohol. A convenient form of apparatus is described by Dakin (see sketch in *J. Biol. Chem.*, 1920, **44**, 499) by the use of which the extraction is made continuous, and the temperature and pressure during the extraction may be varied. By this method the amino-acids separate from their solution in butyl alcohol (saturated with water) in the form of a dry crystalline or granular powder, convenient to handle. From the mixture of amino-acids obtained on hydrolysis, after removal of the mineral acid, the greater part of the monoamino-acid fraction is extracted by butyl alcohol in about 20 hours; the portion not extracted consists of the dicarboxylic acids, the diamino-acids, and part of the glycine. Serine (with other hydroxy acids) appears to be unextracted also. The fractionation of the residue, which is thus practically free from monoamino-acids is a comparatively simple matter; for the separation of the monoamino-acids themselves, recourse must be had to one of the methods available, such as the ester method.

The amino-acids may also be obtained in a solid form by means of the so-called "carbamate method" (Kingston and Schryver,

Biochem. J., 1924, **18**, 1070). The amino-acid solution is diluted with 2 volumes of alcohol, the liquid cooled in a freezing mixture, and finely divided barium hydroxide is stirred in till the solution is strongly alkaline to phenolphthalein. Carbon dioxide is then passed in until the pink colour of the indicator is almost discharged, when the current of gas is stopped and more baryta added; the liquid is stirred mechanically throughout the process. The alternate adding of barium hydroxide and passing of carbon dioxide is repeated several times, after which the precipitated barium carbamates are filtered off in the cold, washed with cold dilute alcohol, then with 95% alcohol, and dried in air. On boiling with water, the barium carbamates are decomposed, yielding barium carbonate and the free amino-acids, which may be obtained in a solid form by evaporating their solution to a syrup, and throwing this into absolute alcohol. The main use of the method lies in the separation thereby of glycine and hydroxylysine, these amino-acids alone giving carbamates insoluble in ice-cold water (see below).

Of the attempts which have been made to fractionate the mono-amino-acids by means of certain of their salts, reference may be made to the method outlined by Town (*Biochem. J.*, 1928, **22**, 1083). The copper salts of the amino-acids are obtained in the form of a dry powder, the drying being carried out by means of pure acetone. In this state, the copper salts are capable of separation into three fractions: (1) soluble in water and in dry methyl alcohol—salts of proline, valine and hydroxyvaline; (2) soluble in water, but insoluble in dry methyl alcohol—salts of glycine, alanine and serine; (3) insoluble or sparingly soluble in water and in methyl alcohol—salts of leucine and phenylalanine. The success of the separation depends upon the complete drying of the mixed copper salts, a process which can only be carried out by the use of pure acetone.

Separation of the Individual Amino-acids from the Various Fractions.—The number of acids to be separated is increased, owing to the fact that some of them, which in their original state in the proteins are optically active, are partly racemised during the process of hydrolysis.

Glycine.—The greater part of this acid, when present in appreciable quantities, separates out in the form of the hydrochloride of its ester, after esterification of the hydrolysis products (see above, p. 629). It is, however, generally obtained in some quantity, even

after this separation, in the lower fractions, from which it can be obtained by reversion into the ester hydrochloride and thus separated from the alanine. Levene (*J. Biol. Chem.*, 1905-6, **1**, 45) has suggested its separation in the form of a picrate. (See also Levene and van Slyke, *J. Biol. Chem.*, 1921, **12**, 285.)

As stated above, glycine is the only non-basic amino-acid which yields a barium carbamate insoluble in ice-cold water. When, therefore, the mixed carbamates, prepared as indicated above, are shaken for 1 hour with a fairly large amount of ice-cold water, the glycine compound remains undissolved, and may be filtered off and decomposed by boiling with excess of water.

A further method for the separation of glycine and alanine has been suggested, depending on the fact that their silver salts show distinctly different solubilities. The solution containing these two amino-acids is boiled with excess of silver carbonate, and cooled; on filtering, the alanine silver salt passes into the filtrate, while the salt of glycine remains with the excess of silver carbonate.

Alanine.—From the lowest fraction this acid can be obtained after separation of the glycine ester hydrochloride by hydrolysis and separation of the hydrochloric acid by boiling with lead hydroxide, when it separates in crystalline form. When present with leucine, etc., in the higher fractions, it remains along with the proline in the mother-liquor, when the acids are subjected to fractional crystallisation from water. The proline also remains in the mother liquor, but can be readily separated from the alanine by extraction with alcohol, in which the former acid alone is readily soluble. It is not easy to obtain the alanine quantitatively from mixtures.

Proline.—This acid is readily obtained from the fractions containing it, owing to the fact that it alone is easily soluble in absolute alcohol. To obtain it, the dried mixture of acid is extracted with 6 times its weight of absolute alcohol. The mother-liquors after the separation of the greater part of the leucine, valine, etc., from aqueous solution are treated in this way, as very little, if any, proline separates in the earlier fractions of crystals. The alcoholic extract is then evaporated to dryness, and re-extracted with alcohol, and, if necessary, these processes are repeated so as to get a more complete separation of proline from the other acids, which dissolve to some extent in alcohol in the presence of proline. The proline thus obtained is a mixture of the optically active and racemic forms. The

copper salt of the former only is soluble in absolute alcohol; the two isomers can therefore be separated from one another. (The amount of proline, with hydroxyproline, in a given fraction can be estimated by reason of the fact that they do not yield nitrogen on treatment with nitrous acid. See details of method of Van Slyke, p. 719.)

It is stated by Kapfhammer and Eck (*Z. physiol. Chem.*, 1927, **170**, 294) that proline and oxyproline alone are precipitated from an amino-acid mixture by Reineck's acid (ammonium chromium thiocyanate).

Town (*Biochem. J.*, 1928, **22**, 1083) has described a method of obtaining very pure proline by a process based on the solubilities of the copper salts of proline and other amino-acids in water, alcohol, and dry methyl alcohol.

Valine, Leucine and Isoleucine.—The separation of these amino-acids from one another is a matter of some considerable difficulty. The proline-free mixture is employed for the separation. By the method of F. Ehrlich and Wendel (*Biochem. Z.*, 1908, **8**, 399), the acids are converted into their copper salts and then extracted with methyl alcohol, which dissolves isoleucine and valine. These two, acids are then liberated from the copper salts by acids, racemised by heating with barium hydroxide in an autoclave to 150°, and the racemised acids are then reconverted into copper salts. These are then extracted with cold methyl alcohol, or hot 96% ethyl alcohol, which removes the isoleucine. This method of separation is not, however, very complete.

Another method has been suggested by Levene and Van Slyke (*J. Biol. Chem.*, 1909, **6**, 391 and 419). This depends upon the fact that the lead salts of leucine and isoleucine are less soluble than that of valine. In the presence of 3% of valine they can be quantitatively separated without any of this substance being carried down. To carry out the separation, the carbon is estimated in the mixture, and a little more lead acetate solution is added than is necessary to precipitate the leucines only, which amount can be calculated from the carbon content of the mixture. The acids are dissolved in 7 times their weight of boiling water, to which solution is added 1.5 c.c. of concentrated ammonia for every gram of acid. Lead acetate in 1.1 molar solution is then added in the quantity of 4 c.c. for each grm. of the leucines. The precipitate thus formed is washed with 90% alcohol. If the ratio of leucine to valine is greater than

2:1 (or the carbon content of the mixture exceeds 53.7%), the leucine is not quantitatively precipitated. A smaller quantity of lead acetate than is necessary for complete precipitation is then added, and the filtrate from the lead salt is concentrated *in vacuo*, made alkaline with ammonia, and the remainder of the leucine is then precipitated by the requisite quantity of lead acetate, or as an alternative, the filtrate from the first lead precipitate can be evaporated to dryness, the carbon content determined in the mixture thus obtained, and the leucine is then precipitated from the solution by the requisite quantity of lead acetate in the presence of ammonia (the amounts of water, lead acetate and ammonia being those given above). Valine is obtained from the mother liquor of the lead salt by precipitating the slight excess of lead with hydrogen sulphide, evaporating the filtrate to dryness, and washing the residue with alcohol-ether mixture (3 parts of alcohol to 1 of ether). To regenerate the leucines, the lead precipitate is dissolved in dilute acetic acid and treated with hydrogen sulphide. The filtrate from the lead sulphide is evaporated to dryness, and washed with a mixture of equal parts of alcohol and ether. The ratio of leucine to isoleucine can be determined polarimetrically, or the acid can be separated by means of the copper salts, that of isoleucine being soluble in methyl alcohol.

A further method for separating leucine has been suggested, depending on the fact that its zinc salt is sparingly soluble in cold water. By boiling the mixed amino-acids with excess of fresh zinc hydroxide, filtering, and evaporating the filtrate, the zinc salt of leucine separates in the form of white crystalline crusts; from these the leucine is liberated by means of hydrogen sulphide in hot aqueous solution.

Phenylalanine.—Phenylalanine ester is readily soluble in ether, and advantage is taken of this fact to separate it from the other esters of the fraction in which it occurs. The fraction is thrown into water, and if phenylalanine ester is present in large quantities it separates in oily drops. In any case the mixture is extracted with ether and the ethereal extract, after distillation of the ether, is hydrolysed with hydrochloric acid, and the phenylalanine can then be obtained in the form of its hydrochloride. Phenylalanine also gives a copper salt which is very sparingly soluble in water, and may be used as a means of separation; leucine, however, gives a copper salt with a similar solubility.

Glutamic and Aspartic Acids.—As stated above, these acids are usually brought down from the hydrolysis mixture in the form of their barium salts; in Dakin's method, they are found in the residue after extraction with butyl alcohol, and may be separated from the other constituents of the residue in the same manner. When the ester method is used, the greater part of the glutamic acid is isolated as the hydrochloride before the esterification of the amino-acids (see above, p. 628). The remainder, together with aspartic acid, is obtained in the fraction from which phenylalanine ester has been removed by ether by the above-described method. This is then hydrolysed by barium hydroxide solution. Barium aspartate (in the racemic form) separates out. From the filtrate from this, glutamic acid can be separated as hydrochloride, by saturating with hydrogen chloride and allowing to stand for some days at 0° . After the removal of glutamic acid as hydrochloride, the filtrate is concentrated under reduced pressure to remove as much hydrochloric acid as possible, and fresh lead hydroxide is added slowly to the boiling liquid until alkaline to litmus, heating being continued for 15 minutes after this point. The liquid is allowed to stand in the cold overnight. Lead aspartate and chloride are thus precipitated; the former has a solubility of 1 in 4,700 in cold water. The precipitate after washing is most conveniently decomposed by means of dilute sulphuric acid; the solution thus obtained contains aspartic, sulphuric and hydrochloric acids, of which the two last-named are easily removed by the usual methods.

The filtrate from the lead salt precipitation is freed from lead by sulphuric acid, and also from small amounts of basic substances that are usually present by means of the phosphotungstic acid reagent, excess of the latter and of sulphuric acid being subsequently removed.

The solution now contains hydroxyglutamic acid (if present among the hydrolysis products). The silver salt of this acid is precipitated by the alternate addition of 15% silver nitrate and normal sodium hydroxide so long as a white precipitate is formed, excess of the alkali being avoided. The free acid is liberated from the silver salt by hydrogen sulphide, and the solution of the acid, on evaporation *in vacuo*, slowly gives colourless crystals of the pure acid (Dakin, *Biochem. J.*, 1918, 12, 290).

Serine.—The ester of this acid is not soluble in petroleum spirit. If the fractions containing it are treated with a little water, and the

mixture then thrown into 5 or 6 times the volume of petroleum spirit, the serine ester is precipitated as an oil which, after again being washed with petroleum spirit, is hydrolysed with barium hydroxide solution. After separation of the excess of barium, the free acid separates out on concentration, and can be purified by washing with alcohol and recrystallisation from water.

Tyrosine, Tryptophane and Cystine.—These three acids cannot be isolated in the form of their esters in the processes described above. The amount of some of these products which can be obtained by hydrolysis can be estimated when only small amounts of the protein are available for investigation. The quantitative estimation of these products will therefore be treated later on, when discussing the routine methods for protein examination.

Tryptophane is destroyed by acid hydrolysis, and can only be isolated when the hydrolysis is brought about by enzymes.

In the above sketch only the outlines are given of the methods employed and suggested for the isolation of the mono-amino-acids from the hydrolysis products. Numerous examples of the applications of the methods are to be found in the literature (*e. g. Z. physiol. Chem.*, 1903, *et. seq.*; *Amer. J. Physiol.*, 1907 *et. seq.*).

B. THE SEPARATION OF THE DIAMINO-ACIDS (HEXONE BASES)

The method at present most widely used for the separation of these basic amino-acids depends upon the fact that arginine and histidine can be precipitated as silver salts, whereas lysine is not so precipitable, and can be recovered from the mother-liquor as picrate or phosphotungstate. The arginine and histidine can themselves be separated by reason of the fact that the silver salt of the latter is precipitated from approximately neutral solutions, and that of the former only from solutions that are alkaline. The method was originally due to Kossel and his pupils (Kossel and Kutscher, *Z. physiol. Chem.*, 1900, **31**, 165; Kossel and Patten, *ibid.*, 1903, **38**, 39; see also Osborne, *Amer. J. Physiol.*, 1908-9, **23**, 180). The method outlined above takes no account of the possible presence of the recently isolated bases, hydroxylysine and protoctine; these are found in the lysine fraction in the above fractionation (see below).

The protein is hydrolysed in the usual way (see above, p. 626), and the bases are precipitated from solution as phosphotungstates, as there described. From the combined phosphotungstates, after

washing, the free bases are obtained by means of barium hydroxide. The phosphotungstates are suspended in water, in as fine a state of division as possible, and are then shaken up, preferably in a mechanical shaker, with excess of finely powdered barium hydroxide till the liquid remains permanently alkaline. The mixture is filtered, and the residue is again shaken with laryta and water; the combined filtrates are freed from barium by sulphuric acid, and the liquid is concentrated *in vacuo* till it contains about 1% of nitrogen (Kjeldahl). The solution is made distinctly acid with sulphuric acid, and 20% silver nitrate is added, any small precipitate being filtered off after allowing to stand for a few hours. (This precipitate will contain silver chloride, and any purine bases that may have been present as impurities.) More silver nitrate is next added, till silver is present in excess; this is shown by adding a drop of the filtered liquid to a drop of clear cold barium hydroxide solution; when a dark brown precipitate is formed, instead of a cream-coloured one, silver has been added in excess. (Instead of silver nitrate, silver sulphate may be used, in which case the nitrogen distribution in the various fractions is more easily determined. Silver sulphate can be added either in hot concentrated solution, or little by little as a solid to the hot solution of the bases, the mixture being well stirred after each addition, till the sulphate has dissolved.)

When silver is present in small excess in the solution, finely powdered barium hydroxide is added in small amounts, until a drop of the clear supernatant or filtered liquid no longer gives a white precipitate with a reagent which is prepared by adding ammonia to 10% silver nitrate solution till the precipitated silver hydroxide has just redissolved. The precipitate at this stage consists of the histidine silver compound; after this has been removed, the arginine silver compound is precipitated by adding excess of barium hydroxide. The filtrate left after removal of the arginine silver contains the lysine; its treatment is described below (p. 641). This procedure gives an approximate separation of the three bases; it should either be repeated on each of the fractions of the silver salts, or the arginine and histidine silver compounds may be precipitated together by adding excess of barium hydroxide, and then separated by one of the methods described below.

Arginine and Histidine.—The precipitate of the mixed silver salts is finely ground up in water containing sulphuric acid,

the disintegration process being facilitated by the addition of silver sand. Enough sulphuric acid should be employed to make the liquid acid. Hydrogen sulphide is then led in, and the liquid is warmed to expel the excess, and then filtered from the precipitated silver sulphide and barium sulphate. The precipitate is thoroughly washed, the washings added to the main filtrate, and the whole is evaporated, and made up to such volume that it contains 1-2% of nitrogen. Histidine is then precipitated by means of mercuric sulphate (10% solution in 5% sulphuric acid), the precipitate is removed and decomposed with hydrogen sulphide, and the histidine present in the liquid is re-precipitated (after removal of excess of hydrogen sulphide) as the silver compound.

The filtrate from the mercury precipitate is freed from mercury and sulphuric acid (by hydrogen sulphide and barium hydroxide, respectively), and excess of silver, as nitrate or sulphate, is added as before. A small further precipitate of histidine silver appears here, and is added to the main histidine fraction. On adding barium hydroxide to the filtrate, arginine silver is precipitated.

The silver salts of both bases are decomposed by passing hydrogen sulphide through their hot solutions in dilute sulphuric acid; after filtering, the excess of sulphuric acid is removed, and the bases are obtained by evaporating their respective solutions (or, if it not necessary to obtain the free bases, the amount of each present may be estimated by determination of nitrogen by Kjeldahl's method).

For further purification of the individual bases, certain derivatives may be prepared. Arginine gives a crystalline compound with flavic acid (see below), a well-defined picrate, m. p., 205-6°, which recrystallises from water, and a nitrate, m. p., 145° (decomp). Histidine gives a picrolonate, m. p. 232° (decomp), by the addition of histidine, in equivalent amount, to picrolonic acid in solution in hot alcohol; the picrolonate is very sparingly soluble in the cold.

Flavic Acid Method.—Recently, a convenient method for the separation of arginine has been introduced, based on the fact that this substance gives certain sparingly soluble salts with flavic acid (1-naphthol-2, 4-dinitro-7-sulphonic acid, "naphthol yellow"). The flavic acid is used as a 5% solution in 5% sulphuric acid, and is added in slight excess to the solution of the bases in the cold; the bases should be in about 10% solution. After vigorous stirring or shaking, the arginine is precipitated slowly as an orange-yellow

deposit (apparently a mixture of mono- and di-flavates). The mixture is allowed to stand in the cold for 24 hours, after which the crystalline deposit is removed and may be recrystallised from 5% sulphuric acid. The flavates are decomposed, in small quantities at a time, by dissolving in 5% sulphuric acid at 60–80°, and extracting the flavic acid with butyl alcohol. After removal of sulphuric acid from the liquid after this extraction, the arginine may be obtained as carbonate on evaporation. The excess of flavic acid in the original filtrate and washings may be removed in a similar manner with butyl alcohol. (See Kossel and Staudt, *Z. physiol. Chem.*, 1926, **156**, 270; Pratt, *J. Biol. Chem.*, 1926, **67**, 351.)

Lysine.—The filtrate from which the arginine and histidine have been removed as silver compounds is acidified with sulphuric acid, and the excess of silver is precipitated by hydrogen sulphide. The filtrates and washings are concentrated to such a volume that the liquid contains about 1–2% of nitrogen, sulphuric acid is added to the extent of about 4% of the total liquid, and then phosphotungstic acid (in 10% solution) until the filtered sample on addition of more reagent remains clear for 10 seconds. After standing for 24 hours, the precipitate is filtered, washed with 4% sulphuric acid, and then decomposed with excess of barium hydroxide. This latter process is accomplished by making the precipitate into a paste with water, and throwing it into boiling water; concentrated barium hydroxide solution is then added in excess (until the liquid is strongly alkaline) and the barium phosphotungstate is filtered off. The excess of barium is then precipitated by carbon dioxide, and the filtrate from the carbonate evaporated down. The residue is taken up with water, and the nitrogen (lysine nitrogen) estimated in an aliquot portion, and again after filtration, evaporated. The resinous-looking residue is then stirred round with an alcoholic solution of picric acid which is added in small quantities at a time. Care must be taken not to add an excess of picric acid, in which the lysine picrate dissolves. The reaction is best carried out in a white porcelain basin. The precipitate, after standing for 24 hours, is filtered off, washed with a little alcohol, dissolved in a small quantity of hot water, the solution is filtered if necessary, and then evaporated till the lysine picrate separates on cooling, in needles. It is collected on a weighed filter, washed with alcohol, dried and weighed. Its formula is $C_6H_{14}O_2N_2 \cdot C_6H_2(O_2N)_3OH$. From the mother liquor

of the picrate, a small quantity of lysine can still be obtained by adding sulphuric acid (to 4%), extraction of the picric acid by ether, and precipitation of the remaining base by phosphotungstic acid; from this the lysine is obtained as picrate by the method already described.

Hydroxylysine; Proctoctine.—These recently-described bases are found with the lysine, when the silver salt method of separation is employed; the former does not yield a crystalline picrate. The solution of the mixed bases (free from picric acid) on treatment by the carbamate method (*q. v.*) and extraction of the mixed carbamates by ice-cold water gives a residue of insoluble carbamate containing the hydroxylysine; the amount of which may thus be estimated.

Proctoctine differs from the other bases found among the hydrolysis products in being appreciably soluble in absolute alcohol; on extracting the mixed bases (which must be free from their carbonates) with this solvent, the proctoctine is removed.

It is needless to add that throughout all the above processes for the separation of the bases, every care must be taken thoroughly to wash the precipitates, which are often very bulky. This is in many cases conveniently done by removing them from the filter and grinding them up in a mortar (sometimes with the addition of silver sand) with the liquid which is used for washing. When an estimation of the amino-acids only is required, it will suffice to determine the nitrogen by Kjeldahl's method in the various fractions, without troubling to isolate the acids themselves in crystalline form.

By no method of separation has a quantitative yield of mono-amino-acids been obtained from a protein, nor is it certain that the whole of the amino-acids coupled up in the polypeptide radicle have as yet been isolated.

Classification of the Proteins

The classifications drawn up in 1907-1908 by the Physiological and Chemical Societies, and by the American Physiological Society, were similar in their main outlines; they are still in use at the present day, although possibly they are no longer really adequate. According to the schemes then adopted, the proteins are divided into three main groups:—I. The simple proteins, whose molecules are composed only of amino-acids; II. the conjugated proteins—simple

of the solution with magnesium sulphate or sodium chloride, or by half-saturation with ammonium sulphate. Animal globulins are very sensitive to physical conditions and can only be obtained in an unchanged condition with difficulty. Some of the albumins and globulins can be obtained in crystalline form.

(e) *The Alcohol-soluble Proteins or Prolamines*.—These proteins are of vegetable origin, and the typical member of the class is gliadin from wheat flour. They were at one time called the gliadins, but Osborne has substituted the name prolamine to indicate that the members on hydrolysis yield relatively large quantities of proline and amide nitrogen. They also yield large quantities of glutamic acid. They are almost insoluble in water and in absolute alcohol, but are easily soluble in alcohol of 70–90% (75% being usually employed).

(f) *The Glutenins*.—Like the prolamines, with which they are always associated, these proteins are only found in the seeds of certain plants. They are insoluble in alcohol-water, salt solutions, or water, but are soluble in dilute alkalis, from which solution they can be precipitated with acid.

(g) *The Scleroproteins* (or, according to the American nomenclature, *the Albuminoids*).—This is a somewhat heterogeneous class of protein substances, derived chiefly from the supporting and connective tissues of animals. The chief representatives of the class are gelatin, hair, horn and keratin.

II. The Conjugated Proteins. (a) *The Nucleoproteins*.—The proteins of this class are widely distributed, and from the fact that they were originally isolated from the fish spermatozoa, which consist chiefly of nucleus, they are supposed to form the chief constituent of nuclei. The protein in the this class is held in some form of combination with nucleic acid (p. 682). Nucleoproteins are found in both plant and animal cells.

(b) *The Glycoproteins*.—The substances of this class are those in which a protein is associated with a carbohydrate group, the latter being easily split off by the action of acids. According to Levene, the prosthetic group contains acetic acid, glycuronic acid and sulphuric acid, and a hexose. He classifies the glycoproteins under two heads:—*mucins*, such as the mucin of the salivary glands and of the alimentary canal, in which the hexose is glucosamine; and *mucoids*, in which the associated group contains galactosamine and chon-

droitin sulphuric acid. The mucoids are widely distributed in the body in the form of cartilage and tendon.

(c) *The Phosphoproteins*.—The chief members of this class are caseinogen of milk and vitellin of eggs. They yield phosphoric acid on gentle treatment with alkalis (1% sodium hydroxide at 37°). They are distinctly acidic in character. The complex containing the phosphoric acid can be split off by the enzyme, phosphoric esterase, of bone, and has been shown to be a peptide-like substance consisting of hydroxyamino-acid residues united together through phosphoric acid residues.

(d) *The Hæmoglobins* and probably other allied substances, such as turicin, which are decomposed on treatment with cold dilute acid into a protein and a chromatogenic group.

(e) *The Lecithoproteins*.—This group includes substances supposed to be compounds of lecithin-like substances with proteins. They are possibly only adsorption compounds.

III. Derived Proteins. (a) *Metaproteins*.—Acid and alkali albumins, substances obtained by gentle treatment of proteins by acid and alkali, which are soluble in excess of both acid and alkali, but are precipitated from solution on neutralisation.

(b) *Proteoses*.

(c) *Peptones*.

(d) *Polypeptides*.

The members of classes (b) and (c) are derived from proteins by enzymatic digestion. They will be treated in detail in a separate section (see p. 706). The peptones are probably in most cases polypeptides, but this latter name is generally reserved for the synthetically obtained substances. Certain soluble proteins undergo change on heating, yielding insoluble so-called *coagulated proteins*. It is not perhaps convenient to place the substances thus produced in a separate class.

Qualitative Reactions of the Proteins

The proteins as a class are recognised with tolerable facility. The following are the chief reactions of analytical value for the proteins.

1. On heating, proteins decompose with evolution of ammonia, and give an odour of burning animal matter. Smaller quantities may be recognised by the ammonia evolved when they are heated

with soda-lime. Sulphur is present in all true proteins, and may be detected by igniting the substance with alkali-metal carbonate and potassium nitrate, dissolving the product in dilute hydrochloric acid, and testing the clear solution with barium chloride, when a white precipitate of barium sulphate will be produced if the substance tested contained sulphur. The sulphur of proteins may also be detected by boiling the substance with a strong solution (33%) of sodium hydroxide and a few drops of lead acetate, when black lead sulphide will be produced.

The foregoing tests merely prove the presence of nitrogen and sulphur, and are not conclusive evidence of the presence of proteins. For the more certain recognition of proteins, the following tests may be employed.

2. The solutions of the proteins have a lævo-rotatory action on polarised light. The optical activity is modified by free acids and alkalis, but not by neutral salts.

According to Gamgee and Croft Hill, haemoglobin is dextro-rotatory (*Ber.*, 1903, **36**, 913). The nucleoproteins are also dextro-rotatory (Gamgee and Jones, *Beitr. chem. Physiol. Path.*, 1903, **4**, 10).

3. The faintly acid solutions of most proteins (not peptones or proteoses) are coagulated when heated to boiling for a few minutes. Very small amounts of free alkali suffice to prevent the reaction, and it is retarded or prevented by any considerable quantity of free acid. Coagulation of proteins (except peptones and proteoses) may also be effected by addition of excess of strong alcohol. The conditions affecting the coagulation are described at length on pages 665 *et seq.*

4. Proteins are precipitated from solution by various mineral acids. Nitric acid is most generally employed. If the acid is allowed to flow into a protein solution, a white ring forms at the junction of the liquids. This reaction is known under the name of *Heller's test*.

Acetic, tartaric, lactic and orthophosphoric acids do not precipitate proteins, but precipitation is caused by metaphosphoric acid.

5. Ferrocyanic acid is a good precipitant of the proteins. If the solution of the protein is rendered distinctly acid with acetic acid, and potassium ferrocyanide added, a white flocculent precipitate is produced. The reaction is delicate, and answers with all varieties of proteins, except certain proteoses and peptones. These substances and gelatin are not precipitated. Recently-prepared hydroferro-

cyanic acid has been recommended in place of acetic acid and a ferrocyanide. A thiocyanate gives a similar reaction.

6. Trichloroacetic acid and sulphonylsalicylic acid are good precipitants of the proteins. The precipitates of certain proteoses produced by trichloroacetic acid dissolve in water on heating, but it must be remembered that the acid can cause a certain amount of hydrolysis. The peptones are not precipitated by trichloroacetic acid.

7. Uranyl acetate precipitates the proteins.

8. Proteins are precipitated by the salts of heavy metals. The precipitates thus obtained have no definite chemical composition, and the phenomena involved in the precipitation are somewhat complex. There appears generally to be a certain amount of formation of a metallic salt of the protein, but complete reaction is inhibited by the absorption of salt molecules from the solution. Precipitation of the protein is ascribed in some cases to the neutralisation of the charge on the colloidal particles of protein by the charge carried by the ions of the salt employed.

9. Proteins are precipitated from solution by salts other than those of the heavy metals. The salt most frequently employed is ammonium sulphate. The concentrations at which precipitation commences and is completed are characteristic of the individual proteins. Salt precipitation is therefore employed for separating proteins from one another.

10. By virtue of their colloidal character, proteins are carried out of solutions by precipitation therein of other colloids. The substances chiefly employed are gum mastic in alcohol and dialysed ferric hydroxide. These colloidal reactions have been extensively employed for the quantitative separation of proteins from solution. Certain powders are also capable under favourable conditions of completely adsorbing proteins from solution. The most effective adsorbents are precipitated silicic acid, meerschaum and iron oxide (Landsteiner and Uhlirz, *Zentralbl. Bak. u. Par.*, 1906, (i), 40, 265).

11. Proteins are precipitated from solutions by the ordinary alkaloidal reagents (see Vol. 7, p. 22).

Those chiefly employed are:

(a) Phosphotungstic acid and phosphomolybdic acid (especially the former).

(b) Potassium-mercuric iodide and potassium-bismuth iodide.

(c) Tannic acid.

(d) Picric acid.

The tannic acid can be employed either in the form of *Almén's reagent*, which is prepared by dissolving 4 grm. of tannic acid in 190 c.c. of 50% alcohol, and adding 8 c.c. of acetic acid of 25% strength, or in the form recommended by *Hedin*, which is made up in the following way: 100 grm. of tannic acid, 25 grm. of sodium acetate, 25 grm. of sodium chloride and 50 c.c. of glacial acetic acid are dissolved in water, and the solution is then made up to 1 litre.

The picric acid is generally employed in the form known as *Esbach's solution*, which is prepared by dissolving 10 grm. of picric acid and 10 grm. of citric acid in water and diluting to 1 litre.

The above alkaloidal reagents precipitate the majority of the proteins in acid solutions only; the strongly basic protamines, however, can be precipitated in alkaline solution. The peptones are not precipitated by picric acid or potassium mercuric iodide (see however p. 704 *et seq.*) but are precipitated by tannic phosphomolybdic and phosphotungstic acids.

Colour Reactions of the Proteins

The most characteristic reactions of the proteins are the colour reactions. These are not common to all proteins, as certain of the reactions are due to special groups, such as tryptophane, which are not found in all members of the class.

The following are the chief reactions employed:

1. Solid proteins are coloured deep yellow by a solution of iodine.
2. Fuming nitric acid destroys solid proteins, with evolution of oxides of nitrogen, but if treated in the solid state with somewhat weaker acid (*e. g.* of sp. gr. 1.2 to 1.25), they acquire a bright yellow colour. The same reaction is produced if the solution of a protein is boiled for some time with strong nitric acid. The colour is attributable to the formation of a yellow substance of indefinite composition known as xanthoproteic acid. On rendering the solution alkaline with ammonia or caustic soda, the colour becomes deep orange. The reaction is due to the presence in the protein molecule of aromatic amino-acids (tyrosine). With gelatin the reaction is negative or faintly positive, this protein containing only traces of tyrosine.

3. When a solution of a protein is treated with Millon's reagent, a white precipitate is formed which turns brick-red on boiling, and the supernatant liquid also becomes red after a time. Solid proteins become red when boiled with Millon's reagent. The reaction depends on the presence of the hydroxybenzene nucleus.

✓ *Millon's reagent* is prepared by treating metallic mercury with an equal weight of nitric acid of 1.4 sp. gr. (or 1 c.c. of mercury to 10 c.c. of nitric acid). When the action slackens a gentle heat may be applied till complete solution is effected. The solution is then diluted with twice its volume of cold water, allowed to stand for some hours, and decanted from the deposit which forms. The liquid thus prepared is a solution of mercurous nitrate, holding nitrous acid in solution, to the presence of which its action is partly due. Hence the reagent answers best when freshly prepared.

Barfoed substitutes for Millon's reagent a neutral solution of mercuric nitrate, which gives a yellow coloration when heated with proteins; and on then adding a drop or two of yellow, fuming nitric acid, and again heating, a bright red or brownish red coloration is produced.

Millon's reaction does not occur in presence of sodium chloride. Gelatin yields the reaction faintly, or not at all.

4. If a few drops of 1% solution of copper sulphate are added to a solution of a protein, followed by addition of excess of sodium or potassium hydroxide, a violet coloration will be produced. If ammonia is substituted for the caustic alkali, a blue solution will be obtained. The violet coloration does not undergo any change on heating the solution to boiling, unless dextrose or other reducing substance is simultaneously present. Fehling's solution may be substituted for the copper sulphate, but in either case excess of copper must be avoided, or the violet colour will be masked by the blue colour of the copper solution.

With certain proteoses the coloration produced is reddish-violet or rose-pink.

The foregoing test is generally known as the *biuret reaction*; according to H. Schiff it is produced by all substances which contain two CONH_2 groups combined in the molecule with one or more CONH groups.

✓ In practice, the biuret test has often to be applied in the presence of neutral salts. Its indications are not affected by sodium

chloride. In presence of magnesium sulphate a white precipitate of magnesium hydroxide is produced by the alkali, and should be filtered off before adding the copper solution. In presence of ammonium sulphate a large amount of sodium hydroxide must be added to obtain the violet coloration.

5. *Colour Reactions Due to the Presence of the Tryptophane Group.*—

(a) The proteins which contain this group give certain definite colour reactions, of which the most important is that due to *Adamkiewicz*. This consists in treating the protein solution with *glyoxylic acid reagent*, and then running in concentrated sulphuric acid, when a strong reddish-violet coloration is produced. The glyoxylic acid reagent is made as follows: 10 grm. of magnesium powder are covered with water, and 250 c.c. of saturated oxalic acid solution are added gradually, the flask being kept cool. The mixture is then filtered, slightly acidified with acetic acid, and diluted to 1,000 c.c.

(b) *Reichl's test* consists in mixing a protein with an alcoholic solution of benzaldehyde, and adding dilute sulphuric acid (equal volume of acid and water) and ferric sulphate. A blue coloration is produced.

(c) *Rhode's Test.*—A weak solution of dimethylaminobenzaldehyde is mixed with the protein; on allowing concentrated sulphuric acid to run in, a reddish-violet colour is produced which changes to dark violet.

(d) *Voisenet's Reaction.*—Protein solutions containing a trace of formaldehyde give a violet colour on addition of saturated hydrochloric acid containing a trace of nitrous acid.

(e) *Romieu* (1925) finds that cell-proteins give a pink colour with syrupy phosphoric acid after gently warming. The test is recommended for histological work.

6. *Reactions Due to the Presence of a Carbohydrate Group.*—The glycoproteins contain a carbohydrate group, in consequence of which they yield certain colour reactions. Other proteins which are not glycoproteins are said to yield the same reactions. It must be remembered, however, that glycoproteins are often only separated with difficulty from other proteins (*e. g.*, in the case of egg-albumin), and conclusions as to the nature of the protein, drawn from the following reactions must be used with caution.

(a) *Molisch-Udransky Reaction.*—Concentrated sulphuric acid is added to a solution of a protein containing a few drops of an alcoholic

solution of α -naphthol. A violet colour is produced, which turns yellow on the addition of alcohol, ether or sodium hydroxide. If thymol is substituted for α -naphthol, a carmine-red colour is produced.

(b) *Bial's* modification of the orcinol reaction: A small quantity of dried protein is added to 5 c.c. of fuming hydrochloric acid, and the mixture is then warmed. When the protein is nearly all dissolved, a little solid orcinol is added, and then a drop of ferric chloride solution. After warming for some time, a green coloration is produced, which is soluble in amyl alcohol.

7. Diazobenzenesulphonic acid in the presence of potassium hydroxide yields an orange to reddish-brown colour which, on treatment with zinc dust, changes to a magenta colour. Tyrosine and histidine yield the same reaction (see p. 699 Estimation of histidine).

8. Ruhemann (*J. Chem. Soc.*, 1910, **97**, 2025) has shown that triketohydrindene hydrate is a delicate reagent for proteins. The reagent is prepared by dissolving 0.1 grm. of the triketohydrindene hydrate in 30-40 c.c. of water. The protein should be in very dilute solution; on heating on the water-bath for 20 minutes with an equal volume of the reagent, a blue colour is produced, showing a broad band in the yellow (see also Harding and MacLean, *J. Biol. Chem.*, 1916, **25**, 337). To get a pure blue colour the protein solution must be accurately neutralised. Some amines and amides give the reaction.

9. On mixing a dilute protein solution with a small quantity of 10% potassium hydroxide solution and adding a drop of 1% solution of diacetyl, a pink colour is developed. Complex proteins, in addition to the pink colour, give a fluorescence which is lost if the protein is allowed to hydrolyse before the diacetyl is added.

10. A 0.01% solution of triformoxime in commercial sulphuric acid gives a pure violet, persistent colour with protein solutions. The test is positive with a 0.05% solution of albumin; pure sulphuric acid gives no reaction (Lewin, 1913).

11. A pink colour is developed by some proteins on heating with sodium hydroxide solution and adding *m*-dinitrobenzene.

12. Some proteins give a reddish-brown colour on warming with a solution of picric acid in dilute sodium hydroxide.

THE GENERAL METHODS OF SEPARATION AND ISOLATION OF THE PROTEINS

The Preliminary Preparation of Materials

As proteins are derived chiefly from animal and vegetable tissues which exist in the form of cells, it is a matter of primary importance to secure a thorough disintegration of material before submitting it to systematic investigation. Unless the cells are completely disintegrated, it is almost impossible to extract the proteins in a satisfactory manner. The difficulties are also increased by the presence of fats and other lipoid matter, which adhere to the proteins and prevent access to them of the solvents employed for extraction. Satisfactory results depend, therefore, to a large extent on the care which has been expended on the preliminary treatment of the materials, the importance of which can be hardly over-estimated.

The method to be employed will depend largely on the nature of the material, and considerable experience and judgment are often necessary to enable the operator to decide on the method to be adopted. For ordinary analytical operations, or for preparations on a small scale, the necessary preliminary processes can be carried out by hand; when a large number of operations have to be carried out, or when it is necessary to prepare relatively large quantities of material (hundreds of grm. or more) it is almost essential to work with mechanical power.

A. Treatment of Material Containing Relatively Large Quantities of Moisture.—Most animal tissues contain about 80% of moisture. Two alternative methods exist for treatment of these and similar materials, which can be submitted to investigation either (a) in the moist state, or (b) after freeing from water and fats and lipoids.

(a) *Treatment in the Moist State.*—The material is first freed from adhering fat (or connective tissue) by dissection, and then coarsely disintegrated by passing it two or three times through a mincing machine. Most of the ordinary domestic implements, such as sausage machines, are available for this purpose. This coarse disintegration leaves most of the cells intact, and these must be broken up before the material is available for investigation. The most usual method of accomplishing this is to grind the material in a mortar with silver sand. When small quantities only are to be treated, this process can easily be done by hand, especially if small quantities of toluene or toluene-water (water saturated with toluene) are added

This hydrocarbon, on account of its capacity for dissolving lipoids, promotes cytolysis. When large quantities of material have to be treated, the grinding process must be carried out with employment of mechanical power, and this is an advantage even when small quantities only are subjected to treatment. A convenient form of pestle and mortar for this purpose is driven by an electric motor which may also serve to drive a large coffee-machine (for grinding dry material) which is mounted on the same table as the mill. The grinding of material is continued until a fine emulsion is formed, which can be readily pressed through muslin. In this condition it is available for extraction of the proteins.

Another method for breaking up the cells consists in alternately freezing and thawing the coarsely disintegrated material. The most effective agent for this purpose is liquid air.

(b) *Treatment by Desiccation*.—Various methods of desiccation have been proposed in recent years, by means of which the material to be investigated can be obtained in the form of a fine dry powder, capable of passing through a fine sieve, and in which the proteins exist in an unaltered condition. As the albumins, on heating, undergo coagulation and other changes, it is necessary that the desiccating processes should be carried out at a relatively low temperature (not exceeding 35–38°).

A process for this purpose has been described in detail by Wiechowski (*Beitr. z. physiol. u. pathol. Chem.*, 1907, **9**, 232), in which the manipulation is not difficult, and which yields a very satisfactory product. For the purposes of disintegration for subsequent protein extraction, Wiechowski's method can be simplified in various details. The material is first passed through a mincing machine with the addition of toluene, and this process can be repeated two or three times. A quantity of water can be extracted from this minced material by suspending it in a linen bag, or metal basket, in toluene in a vessel at the bottom of which is placed some fused calcium chloride; care must be taken that it does not come in contact with the calcium chloride. This preliminary drying is not, however, in all cases necessary. When the finely minced material is moist with toluene, the greater part of the moisture can be readily driven off at a temperature of 37°, especially when the mixture is exposed to a rapid current of air. With small quantities of soft material, such as mucous membrane, pancreas gland, etc., this drying process can

be carried out in an incubator, without the employment of a forced draught. In this case the toluene tissue emulsion is spread in a thin layer on a glass plate. As soon as the surface is dry, the plate is removed from the incubator, and the material is scraped off from the plate, so that the still moist under-surface can be exposed. If necessary, a little more toluene is sprinkled over the moist surface. The material is then returned to the incubator, and the drying is continued until a product is obtained which can be easily disintegrated on grinding in a mortar.

When dealing with harder material, such as muscular tissue, or with large quantities, it is necessary to dry the toluene emulsion of the substance under investigation in a good current of air. For this purpose a special apparatus is necessary, which can, however, be readily constructed by a tinsmith. This consists of a tinned iron chamber, either cylindrical or rectangular in shape, provided with an electrical fan at one end by means of which air can be either sucked or driven through the chamber through a large outlet at the other end. When air is sucked through (and the ventilating fan is placed in the front of the chamber), the air inlet should be covered with a thin sheet of cotton-wool to filter off the dust; where air is driven through (and the ventilating fan is placed at the back of the chamber), the air is filtered through a screen placed just in front of the fan. The toluene emulsion of the substance to be dried is introduced into the front of a chamber on perforated tinned trays, on which it is spread in thin layers. The larger the perforations of the trays, the greater the surface of the mixture exposed to the current of warm air. The air is warmed by placing one or more lighted burners at the back part of the apparatus, some little way behind the trays containing the mixture to be treated. Where a warm (incubator) room is available, the heating is not necessary and the whole drying apparatus can be placed bodily in the warm chamber. When, however, an incubation room is not available, the cylindrical form of the apparatus is preferable, and the air can be best drawn over by suction, the ventilating fan being made so as to fit just inside the front part of the cylinder. A thermometer should be inserted just over the trays containing the material and the heating of the back part of the apparatus can then be so regulated that the temperature does not rise above 37° . The evaporation of the water and toluene serves, however, automatically to keep the temperature low, and

it is only when the material is nearly dry that there is any danger of a large rise of temperature. Even then, the danger of injury to the material by heat is considerably diminished, as proteins have less tendency to undergo change when the amount of moisture present is small.

Various forms of drying chambers for use in an incubation room are illustrated in the catalogues of apparatus manufacturers, but in most cases a simple and inexpensive chamber can be readily designed to meet the particular requirements for any given investigation.

When the drying process is sufficiently complete, the material can be readily disintegrated by grinding. With relatively small quantities of material this process can be easily effected by an ordinary hand coffee-mill, but with larger quantities, it is again advisable to use motor power or a larger and stronger mill either hand or power-driven. Various forms of paint-mills can be employed for this purpose, but the less expensive coffee-mill is generally quite satisfactory, unless large quantities of material are being continually manipulated, under which circumstances a machine of stronger construction is required.

The powder thus obtained generally contains appreciable quantities of fats and lipoids, which can be extracted by an organic solvent either by percolation, extraction in a Soxhlet apparatus, or any other convenient method. After freeing from the solvent, the powder can be sieved. By this process muscular tissue can be separated from the greater part of the fibrous connective tissue, and can be obtained in a form which will pass through a fine-mesh sieve. The enzymes contained in the original tissue also remain intact. When in this form, furthermore, the proteins can generally be readily extracted, as water or other solvents have free access to the solid matter.

Another process for desiccation of tissues which can also be conveniently employed in a large number of cases, consists in the treatment of the material with an anhydrous salt, which by abstracting water is readily converted into a hydrated form. The salts usually employed for this purpose are sodium and calcium sulphates. The former is more convenient, as only a slight rise of temperature occurs when it is converted from the anhydrous into the hydrated form. The tissue or other material is ground up with a little more than equal

weight of the anhydrous salt. The mixture, after standing for some time, can be readily ground to a fine powder. If it appears moist after standing for an hour or so, and cannot be easily pulverised, a little more sodium sulphate should be added. Animal fluids such as blood or serum can also be treated in this way. Njegovan (*Biochem.*, Z., 1912, 43, 203) recommends that in carrying out this process, the material warmed to 37° should be mixed with exactly the amount of anhydrous sodium sulphate necessary to combine with the water (to form $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$), and the mixture should then be dried in a desiccator *in vacuo* over sulphuric acid. The total bulk can, by this means, be kept small. It must be remembered that any aqueous extracts of a powder obtained in this way will contain sodium sulphate in solution, and this fact must be taken into account when it is necessary to precipitate the dissolved proteins by addition of salts. (See *Salt Precipitation of Proteins*, p. 665. The method has been found very convenient when it is required to separate the coagulable proteins by heat precipitation.

As to the relative advantages of examining material in the moist and desiccated conditions, it must be remembered that proteins can be kept for an almost indefinite time without putrefying when in the latter condition. When, therefore, a large number of investigations have to be carried out, it is not always convenient to examine all the samples when fresh. As a large number of samples can be readily desiccated at one time with the apparatus described, the desiccation method will be found to be of considerable service, especially as it is possible to reserve samples for future reference.

B. Treatment of Material Containing Relatively Small Quantities of Moisture.—In this category are included a large number of plant seeds, roots, etc., and also a large number of animal proteins belonging to the group of the scleroproteins. In this case preliminary treatment is comparatively simple. The material is ground in a convenient form of mill (either of the edge-runner or paint or coffee-mill variety) to a fine powder. The form of mill to be employed can be determined by experiment. Small seeds and such like material can be best ground in a mill of the coffee-mill type, whereas large seeds, with husks that are readily broken are easily ground in the edge-runner type of mill or by pestle and mortar. Very hard material, such as horn, must be pulverised by some form of apparatus such as is employed for rock-crushing. When the ground

material contains oils or fats, these should be extracted after grinding, by petroleum spirit or ether. After extraction the material is filtered off from the solvent (conveniently on a Buchner funnel, or by means of a porcelain centrifugal machine) and freed from the last traces of solvent by air-drying. It can then be sieved. In the case of many materials, the greater part of the husks can be separated by the last-named process.

C. Material Requiring Special Treatment.—Certain material which undergoes rapid change requires special treatment. Included in this category is muscular tissue, which rapidly undergoes change after removal from the living organisms, with the onset of rigor. In investigating the protein changes in this case (which have as a rule already taken place in ordinary butcher's meat), it is necessary to remove the tissue as quickly as possible from the animal after death, and to carry out the disintegration process at a very low temperature. Investigations involving treatment of this description are, however, of a very special character, and need no prolonged discussion in this place. Special apparatus has been devised for the purpose (see for example, Kossel, *Z. physiol. Chem.*, 1901, **33**, 5).

In some cases it is convenient to remove as much as possible of substances other than proteins from the material before the isolation of the proteins is attempted. As an example, the preparation of oat glutelin from oatmeal may be quoted (Schryver and Buston, *Proc. Roy. Soc.*, 1926, *B*, **99**, 476). The oatmeal is first treated with taka diastase, whereby the starch is converted into soluble sugars, and removed, whilst the residue, consisting of the proteins together with cellulose, etc., is then treated with 0.2% sodium hydroxide solution, which extracts the protein. Sodium hydroxide, when added to the original oatmeal, gelatinises the starch, and renders filtration almost impossible—a difficulty which does not arise when the starch is thus removed in a preliminary treatment before extraction. The great decrease in the bulk of material to be handled is, of course, also an advantage.

Quantitative Estimation of Total Proteins

A. Estimation of Materials in Which the Only Nitrogenous Products are Proteins.—Where the only nitrogenous products are proteins, the amount of the latter substances present can be estimated with a fair degree of accuracy by ascertaining the amount of nitrogen pres-

ent in the material. When nitrogenous substances other than proteins are also present, the latter must be separated from the mixture by some method of precipitation; the quantity of protein present can in this case be ascertained either by direct estimation of the nitrogen in the precipitate, or by estimation of the total nitrogen in the material, and of the nitrogen in the filtrate from the protein precipitate, the difference between these two numbers giving the amount of nitrogen in the proteins; the method to be chosen will depend upon the relative amounts of the various nitrogenous substances present, or the manipulative details of the experiment.

The nitrogen in the majority of proteins varies within comparatively narrow limits (see p. 692). An approximately accurate estimation of the protein can, as a rule, be obtained by multiplying the amount of nitrogen found by the factor 6.25. If, however, a greater degree of accuracy is required, the pure proteins can be isolated by one of the methods described in the succeeding sections, and the amount of nitrogen in the product thus obtained can be directly estimated. From this result the correct factor can be calculated.

The Estimation of Nitrogen by Kjeldahl's Method.—Nitrogen in proteins is now almost always estimated by the method originally devised by Kjeldahl. This consists in destroying the material by means of concentrated sulphuric acid. The carbon is oxidised to carbon dioxide, and the nitrogen is converted (except in the case of nitro-derivatives and a few other substances) into ammonia. By making the incineration mixture alkaline, after the destruction of the organic substances, and distilling off the ammonia into a known volume of standard acid, the amount formed in the process can be ascertained; from which result, the quantity of nitrogen present in the material can be directly calculated.

Many modifications of the original Kjeldahl method have been described. A large number of these are of quite unessential character, and it is unnecessary in this place to refer in detail to the literature of the subject. The method described below gives accurate results, and affords no difficulties in the manipulation. (For the official American method, see Vol. 1, p. 60.)

A quantity of substance varying in weight from 0.2 to 5 grammes, according to the amount of nitrogen present, is introduced into a round-bottomed flask of hard Jena glass; about 100 c.c. of water, 20 c.c. of concentrated sulphuric acid, and a crystal of copper sulphate

are introduced. In some cases, the addition of sodium sulphate is advisable, in order to increase the temperature of boiling, and thus to hasten the reaction. The flask is then adjusted obliquely over a gas flame, and the excess of water is gently evaporated. The object of starting the process with dilute, instead of concentrated, acid is that the dilute acid hydrolyses the proteins present; the hydrolysis products are more readily destroyed by the concentrated acid than are the unhydrolysed proteins. The copper sulphate promotes catalytically the oxidation process; mercury can also be used for the same purpose. After the water has been boiled off, the flame is kept low, so that frothing is minimised. When the first action is over and the mixture is well charred, the size of the flame can be increased until the liquid in the flask boils briskly. The heating is continued until the contents of the flask are colourless. Care should be taken to have sufficient sulphuric acid present. If the contents of the flask show a tendency to become dry, more concentrated acid should be added. When the material is very resistant, the addition of potassium persulphate has been recommended.

When the incineration is complete, the mixture is diluted with water to about 250 c.c., and, after cooling, 40% sodium hydroxide solution is carefully added until the mixture is strongly alkaline. When copper sulphate has been added, this point will be indicated by the deep blue colour of the liquid. In place of 40% caustic soda solution, solid ("heavy") magnesium oxide may be added in excess, as will be indicated by some of the oxide remaining undissolved in the liquid. In this case, the oxide should be added carefully, with frequent shaking, while the mixture is kept cool. In either case, the mixture is then distilled until the distillate is no longer alkaline. The latter should be collected in a known volume of standard ($N/10$) sulphuric acid, which should be more than sufficient to neutralise all the ammonia which distils over. This amount can be ascertained by titrating the excess of acid when the distillation is complete.

If the incineration has been carried out in a flask sufficiently large (500–700 c.c.) the ammonia can be distilled off from the same vessel. In conducting the distillation, it is advisable to cool the distillate, as the hot vapours may act upon the glass, and too high results are obtained. Various forms of apparatus appear in the catalogues of manufacturers; most of them are in the form of batteries, so devised that several estimations of nitrogen can be carried

out at one time. The form devised by Dr. Horace Brown is convenient, and is so constructed that the vapours exert the minimum action on the glass.

For titrating the excess of acid which has not been neutralised by the ammonia, various indicators can be employed. Dialysed litmus, methyl red and sodium alizarin sulphonate give satisfactory results.

Modifications of Kjeldahl's method have been devised to meet the cases where nitro-derivatives or other substances in which the nitrogen is not converted by sulphuric acid into the form of ammonia. Such substances are, however, rarely present in the material in which proteins have to be estimated, and it will suffice in this place to indicate the most commonly employed process used in these cases, viz., the method of Jodlbauer. The substance, if not in solution is intimately mixed with calcium sulphate (plaster of Paris) and introduced into the incineration flask. 25 c.c. of a mixture made by dissolving 40 grm. of phenol in 1 litre of concentrated sulphuric acid are then introduced and shaken round for a few minutes with the mixture of the substance with plaster of Paris. After standing for 5 minutes and cooling, 2-3 grm. of well washed zinc dust and one or two drops of mercury are introduced. Heat is gradually applied, and the heating is continued until the mixture is colourless. The ammonia is then distilled off in the usual way. In these operations the phenol is converted into a nitro-derivative, which is then reduced to an amino-derivative by the zinc. From the amino-derivative, ammonia is formed by the subsequent action of the sulphuric acid.

Micro-method for Estimation of Nitrogen (Micro-Kjeldahl).—In cases where little material is available it is of advantage to use a micro-method for the estimation of nitrogen. The results given are quite accurate, and the method is specially valuable when working with solutions of proteins and other nitrogenous materials. In using the method for estimating the nitrogen in solids, it is best to weigh out a suitable amount of substance, dissolve this in a known volume of an appropriate solvent, and to estimate the nitrogen in an aliquot part of the solution. A suitable solution should contain from 0.4-0.8 mg. of nitrogen per 1 c.c.

1 c.c. of the solution, together with 1 c.c. of pure "nitrogen-free" sulphuric acid, a drop of saturated copper sulphate solution, and a small crystal of potassium sulphate, are heated together in a hard-glass test-tube (8 in. by 1 in.) over a small "arsenic" burner. A

3 in. funnel, inverted over the mouth of the tube, and connected with a filter-pump, serves to draw off the fumes of sulphur trioxide, etc. Incineration is usually complete in 10–20 minutes, and will be shown by the liquid becoming quite clear. After cooling, the clear liquid is washed into the distillation-chamber of the apparatus shown in the figure; it is made alkaline with 10 c.c. of 40% sodium hydroxide

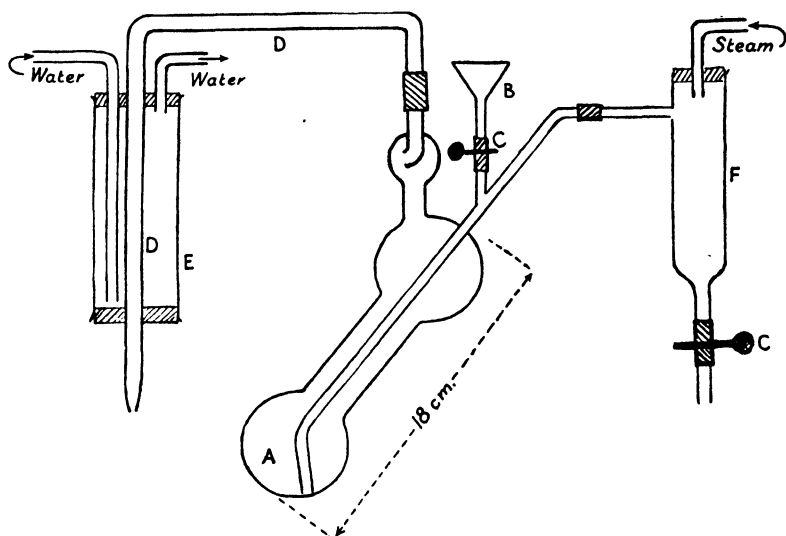


FIG. 33.—Distillation apparatus for *Micro-Kjeldahl* estimations.

- A—Distillation-chamber (80 c.c. capacity)
- B—Funnel
- C—Clip
- D—Wide tube of Silica or pyrex glass
- E—Water jacket
- F—Trap-vessel (100 c.c.) into which residual liquid from A returns on stopping current of steam.

solution, and the mixture is distilled by means of a rapid current of steam. The ammonia liberated is collected in 5 c.c. of $N/70$ sulphuric acid, methyl red being used as indicator. Distillation is continued for 3 minutes while the jet of the delivery tube dips under the surface of the acid in the beaker, and for a further 1 minute with the tube raised above the acid. The excess of acid is titrated with $N/70$ sodium hydroxide (5 c.c. of $N/70$ acid or alkali are equivalent to 1 mg. of nitrogen). In making up the acid and alkali, the former should be exactly $N/70$, and the latter of such a strength that

5.05 c.c. are equivalent to 5.00 c.c. of acid; the difference is ignored in titrating, and is in the nature of a "blank" for the reagents employed.¹

The micro-method is very rapid in practice, several determinations being possible in an hour.

B. Estimation of Proteins in Materials in Which Nitrogenous Substances Other than Proteins are Present.—A very large number of methods have been proposed for estimating proteins when these substances are present with other nitrogenous compounds. The following are the chief processes employed: (a) precipitation by compounds of heavy metals; (b) precipitation by organic liquids especially alcohol; (c) precipitation by metallic salts; (d) precipitation of coagulable proteins in the form of a coagulum, (e) precipitation by or absorption from solution by colloids; (f) precipitation by alkaloidal reagents. The choice of method to be adopted will depend on various circumstances. Thus, for example, all proteins are not coagulable; if, therefore, a coagulation method be applied to materials containing substances of this character, the total proteins will not be precipitated. Various tissue extracts and animal fluids contain, in addition to coagulable proteins, such substances as mucins and nucleoproteins; these latter compounds will not be carried down quantitatively in the coagulum. In this case an alcohol precipitation method can be employed. It is possible, however, that the coagulation method may in this case be used to separate the coagulable from the non-coagulable proteins. (See, however, the discussion of the coagulation method given below) (p. 666.)

Where proteins occur mixed with their more complex digestion products, their separation from these latter substances is a matter of some difficulty. Certain of the proteoses (albumoses) are precipitated by the ordinary protein precipitants, such as alcohol, salts etc., whereas others are not. In these cases, where possible, the proteins proper should be separated by a coagulation method. Animal tissues, as a rule, are free from proteoses, but certain natural vegetable products contain them. It is doubtful whether in such cases a perfectly satisfactory method exists for the estimation of the total protein apart from the digestion products.

(a) *Precipitation with Compounds of Heavy Metals.*—*The Process of Stutzer.*—This method consists in precipitating the protein with copper hydroxide. The reagent is prepared, according to Stutzer, by

¹ The apparatus must be steamed out thoroughly before use.

the following method: 100 grm. of copper sulphate are dissolved in 5 litres of water to which 2.5 grm. of glycerol are added. From this solution the copper hydroxide is precipitated by dilute sodium hydroxide solution which is added until the liquid is strongly alkaline. The copper hydroxide is filtered off and rubbed up with water containing 5% of glycerol, and the supernatant liquid is then filtered off. This washing is repeated until the liquid is free from excess of alkali. The residue is then made up to a paste with water containing 10% glycerol, and so diluted that a paste suspension is formed, which can be readily drawn up by suction into a measuring pipette. The reagent should be preserved in flasks (preferably not too large; Sulzer recommends those of 150 c.c. capacity) filled to the top and tightly corked and kept in a cool dark place. The content in copper hydroxide of the reagent should be determined before use by estimating the total solids. The precipitation of the protein is carried out in the following way: 1 to 2 grm. of the finely sieved material are placed in a beaker with 100 c.c. of water and heated to boiling. Where starch is present, the heating should be continued for 10 minutes, for which purpose the mixture is placed on a water-bath. 0.3 to 0.4 grm. of copper hydroxide is then added and, after cooling, the precipitate is poured into a filter and thoroughly washed with water. The filter-paper and its contents are then transferred to a round-bottomed flask, and the nitrogen estimated by Kjeldahl's method.

This process has been largely applied to the estimation of proteins in plant materials.

When phosphates are present they should be precipitated by the addition of a few c.c. of an alum solution before the addition of the cupric oxide. When insoluble alkaloids are present, they can be removed by heating the material to boiling, first with alcohol containing 1% acetic acid, and then pouring off the supernatant liquid on to a filter, which is afterwards used for filtering the protein precipitate. The residual solid is then treated with water and copper hydroxide in the way already described.

A modification of Stutzer's process, suggested by Barnstein, is an application of a method which was originally employed for the estimation of proteins in milk. 1 to 2 grm. of the material under investigation are heated with 50 c.c. of water to the b. p. When starch is present, the heating is continued for 10 minutes on

the water-bath. 25 c.c. of a copper sulphate solution containing 60 grm. of the crystalline sulphate in a litre are added, and then 25 c.c. of sodium hydroxide solution containing 12.5 grm. of the hydroxide in a litre (with constant stirring). The precipitate is allowed to settle, washed by decantation, the wash waters being poured through a filter and then brought on to the same filter and washed with warm water until the filtrate is free from copper (no reaction with potassium ferrocyanide). The nitrogen is then determined by Kjeldahl's method in the precipitate. By using the quantities of copper sulphate and sodium hydroxide given above, a green basic cupric sulphate is precipitated. Modifications as regards these quantities have been suggested, but it does not appear that the analytical results are more satisfactory. Proteoses are precipitated by the above processes. As peptones do not occur (or only very rarely) in plants, the Stutzer and Barnstein methods can in most cases be applied to the estimation of the total proteins in material of vegetable origin.

Other compounds of heavy metals, such as uranium acetate, have been suggested for protein precipitation.

(b) *Precipitation by Organic Liquids*.—This method is especially valuable for estimating the total proteins in animal tissues, which contain, in addition to coagulable proteins, substances such as mucin or nucleoproteins, which do not coagulate on heating. Alcohol is the solvent most generally employed, and acetone is of frequent use. The solution of the proteins is thrown into from 5 to 10 times the volume of alcohol or acetone, with continual stirring, and, after the mixture has stood for a short time, the proteins generally separate out in a flocculent form which can be readily filtered off, and washed with alcohol of the strength in which the precipitation was carried out (*e. g.*, 5 to 10 times the volume of alcohol to one volume of water). By this method, not only the natural proteins, but also some of the more complex digestion products (proteoses) are also precipitated. The coagulable proteins which are soluble in water gradually become insoluble after long contact with alcohol or acetone. Hence, if it is necessary to redissolve the precipitate, the filtration should be carried out as soon as possible after filtration.

Hardy has employed this process for separating the total proteins from blood serum and plasma. For this purpose the serum or plasma is cooled to 0°, and thrown into several volumes of alcohol or acetone,

cooled to -8° . The filtrate is then filtered off at 0° , washed with cooled ether (0°) until free from alcohol or acetone, and finally extracted with warm ether in a Soxhlet apparatus. When in this form, the warm ether does not coagulate the proteins. The white powder is finally dried *in vacuo* over sulphuric acid, and the total proteins are thus obtained free from adhering lipoids. The advantage of this method is that the proteins can be obtained unchanged, and weighed, and used for subsequent investigations (*Proc. Physiol. Soc., J. Physiol.*, 1910, 40, 68).

(c) *Precipitation by Salts*.—Proteins are precipitated from solution by various salts, of which the chief employed for this purpose are sodium chloride, magnesium sulphate, ammonium sulphate, zinc sulphate and sodium sulphate. The proteins differ from one another in that they are precipitated, either completely or partially, at different degrees of saturation of the solution by the salt. The differential salt precipitation is the only known method by means of which soluble proteins can be separated from one another. As, therefore, salt precipitation is employed more usually as a method for the separation of the individual proteins from one another, rather than as a general method for precipitation of total proteins, it may be more suitably discussed later under the heading of the Systematic Separation of the Proteins (see page 670). With certain limitations it may be employed for the estimation of individual proteins, such as the albumins and globulins of blood serum.

(d) *Precipitation of Coagulable Proteins in the Form of a Coagulum*. The actual changes involved in the process of coagulation are not yet fully understood. Research has, however, clearly indicated that two distinct sets of phenomena are involved in the reaction, *viz.*, (a) a chemical change caused by the interaction of the protein and the water, and (b) the agglutination of the chemically charged protein particles to form a coagulum which can be readily filtered. The former of these phenomena is a chemical reaction, with a high temperature coefficient; the reaction is influenced by the acidity of the medium (the *pH*), the rate of reaction being at a maximum at the iso-electric point of the protein. The second stage, agglutination, is a physical change, and involves the precipitation of the "denatured" protein. The protein exists in solution as an electrically charged colloid; on neutralisation of the charge, the protein is precipitated as

a coagulum. This precipitation is considerably influenced by the presence of salts in the solution, and is only complete at the iso-electric point of the protein. For the majority of coagulable proteins, the iso-electric point is slightly on the acid side of neutrality; it therefore follows that coagulation is best carried out in a faintly acid medium. In media up to the strength of $N/50$ acetic acid, proteins can be heated without hydrolysis. Good results have been obtained when the liquid under investigation has been made acid before heat-coagulation by means of a weak acid, such as butyric acid. This should be added to the mixture until it is distinctly acid to litmus. As the addition of slight excess of weak acid does not largely increase the hydrogen ion concentration (beyond the range for the optimum condition) such weak acids as butyric acid give particularly favourable results. (For a summary of the present knowledge concerning denaturation and coagulation, see "*The Chemistry of the Proteins*" by Dorothy Jordan-Lloyd, 1926.)

Various modifications of the method of simple coagulation in acid solution have been suggested, of which two are given below.

Devoto's Method for Estimation of Proteins in Urine.—100 c.c. of the urine are employed, to which 50 c.c. of ammonium sulphate crystals are added, with continual stirring. The mixture is slightly warmed to promote the solution of the crystals. The mixture is then heated for $\frac{1}{2}$ hour in a closed vessel in a boiling water-bath. The nitrogen is estimated in the coagulum.

Hopkins has modified Devoto's method by filtering the coagulum on to a hardened paper, and then transferring it back to a beaker with water, and heating the suspension for $\frac{1}{2}$ hour in a water-bath. The precipitate is then filtered again on to the same hardened filter. The large amount of salt appears to interfere with the agglutination of the protein.

Coagulation by Alcohol.—A convenient method for estimating proteins in tissues, serum, etc., consists in treating the liquid or solid material in a fine state of subdivision with somewhat more than an equal weight of anhydrous sodium sulphate, which combines with the water, forming hydrated sodium sulphate. After drying in this manner, the mixture can be powdered, and in this form boiled for half an hour under a reflux condenser with absolute alcohol. The alcohol is then filtered off through a nitrogen-free filter paper, and the coagulated protein is washed on to this paper with hot water,

and the washing continued until the washings are free from sulphate. The filter-paper with the coagulum is next transferred to a flask, and the nitrogen is estimated by Kjeldahl's method. In the case of tissues and serum about 5 grm. are sufficient. The tissue or serum is introduced into a glass mortar, the sulphate is added, and after standing for some time, until the material is dry on the surface, the mixture is stirred with the pestle, and, if necessary, more sulphate is added. The addition of this salt should be continued until the whole mass can be readily powdered. It is then transferred to a flask into which also the small amounts of powder remaining in the mortar are quantitatively removed by rubbing them up with small additional quantities of sodium sulphate. After heating with alcohol, the latter is decanted off through the filter, and the residual coagulum is gradually transferred to the filter by means of hot water. The nitrogen can, if necessary, be estimated in the filtrate instead of the coagulum. The former method is, however, the more convenient, as the coagulum is free from salt and can be incinerated more readily with smaller quantities of sulphuric acid. Mucins and nucleoproteins will be found in the filtrate from the coagulum, but no experiments have been carried out to determine whether the method is available for separating quantitatively in all cases such substances from coagulable proteins. It is, however, applicable to the estimation of total proteins in a large number of materials, and has, furthermore, the advantage that where a large number of analyses have to be carried out with material that readily putrefies, the latter can be kept after treatment with sodium sulphate for a long time without undergoing change, and the analyses can be done when convenient. The amount of non-protein nitrogen can be ascertained in the filtrate of the coagulum, or, better still, by estimating the total nitrogen in a fresh portion of the material, and deducting therefrom the amount of nitrogen found in the coagulum.

(e) *Separation of Proteins from Solution by Precipitation with, or Adsorption by Colloids.*—Certain colloidal substances, such as kaolin, adsorb proteins from solutions, and the method has been employed for quantitatively separating proteins from solution. There have been introduced other methods depending on the capacity of colloidal substances for removing proteins from solution, in one of which gum mastic is employed, and in the other, dialysed colloidal ferric hydroxide.

Gum-mastic Method. (*Biochem. Z.*, 1906, 2, 219; 1907, 3, 109; 1907, 5, 365; 1907, 6, 1.)—When an alcoholic solution of gum-mastic is added to water, an emulsion is formed, which can be de-mulsified on the addition of electrolytes, when the mastic separates in the form of a flocculent precipitate. If proteins be present at the same time, these will be carried down with the mastic precipitate, if the latter be present in sufficient excess. Certain of the proteoses are also precipitated in this way. The method has been chiefly applied to blood serum, and the method of employment is illustrated in the following example: One volume of serum is mixed with three volumes of alcohol, whereby a part of the protein is precipitated. To the filtrate is then added one volume of a 50% solution of mastic in alcohol. The mixture is diluted till the alcohol does not form more than 30% of the total fluid. The liquid is then just acidified with acetic acid, and 10% magnesium sulphate in the proportion of 10 to 15 c.c. for each litre of liquid is added. The mastic will carry down the last traces of protein. If the total nitrogen in the serum is estimated, and also the nitrogen in the filtrate, the amount of protein can be calculated. It is advisable to separate the proteins partly, by alcohol before addition of the mastic solution, as the latter must be in large excess.

Dialysed Ferric Hydroxide Method. (*Biochem. Z.*, 1908, 7, 329.)—The principle of this method is similar to that of the mastic method, and has also been applied chiefly to the investigation of serum and plasma, although it is applicable to other fluids. As an example of the method, the following illustration is given: 50 c.c. of serum are diluted with 10–12 times the bulk of water. 40 c.c. of *liquor ferri dialysatus* are then added, drop by drop, with constant shaking. The protein is precipitated, the non-protein substances remaining in the filtrate, the presence of salts causing the iron hydroxide to separate from the solution, carrying with it the proteins.

(f) *Precipitation by Alkaloidal Reagents.*—Proteins can be precipitated by potassium mercuric iodide, tannic acid, phosphotungstic acid and other alkaloidal precipitants. The majority of these reagents precipitate many substances other than proteins, so that their use is somewhat limited. Special mention may, however, be made of the following, on account of their usefulness:

Trichloroacetic Acid.—This has been used by Hiller and Van Slyke (*J. Biol. Chem.*, 1922, 53, 253) for the precipitation of proteins from

liquids containing also their degradation products. Trichloroacetic acid, at concentrations of less than 5%, precipitates the whole of the proteins, but permits the amino-acids, proteoses and almost the whole of the intermediate degradation products to pass into the filtrate. The technique is as follows: To a 1% solution containing the protein (etc.) is added, slowly and with constant stirring, an equal amount of a 5% solution of trichloroacetic acid, the resulting liquid thus containing 2.5% of trichloroacetic acid. The mixture is allowed to stand for 30 minutes, and then filtered through a dry folded filter. Filtration is rapid, and the filtrate water-clear. The precipitate is washed on the filter with 2.5% trichloroacetic acid solution.

Tungstic acid is of value when dealing with materials containing only proteins and their digestion products. It precipitates the whole of the proteins and the majority of the intermediate products, but not the simpler peptides or the amino-acids. The reagent is used as follows:—50 c.c. of the solution containing 4% of nitrogenous material are placed in a flask (500 c.c.) and 300 c.c. of water and 50 c.c. of 10% sodium tungstate are added. After mixing, 50 c.c. of $2/3N$ sulphuric acid are run in, and the liquid is made up to 500 c.c. The mixture is filtered after standing 10 minutes. Filtration is rather slow, and the precipitate is bulky. The precipitate is washed with 1% tungstic acid solution in dilute sulphuric acid.

Esbach's Method for Estimating Urinary Proteins.—

This is only a rough method, but it is sufficiently accurate for many clinical purposes and, owing to the simplicity of the manipulation, is well adapted for the purpose for which it was devised. It consists in adding a picric acid solution to urine and measuring in a special tube the amount of precipitate. The picric acid reagent is made by dissolving 10 grm. of picric acid and 10 grm. of citric acid in water and diluting to a litre. The urine is acidified with acetic acid, if not already acid, and diluted till the sp. gr. is 1.006–1.008. This is poured into the tube (Fig. 34) to the mark U. The reagent is then added to the mark R. After standing for 24 hours, the height of the deposit in the tube, which is graduated in amounts of protein in grm. per litre, is read off. The method can also be employed for approximate estimations of protein from other sources.



FIG. 34.
Esbach's
tube.

THE ISOLATION OF THE INDIVIDUAL PROTEINS FROM THE PREPARED MATERIAL AND THEIR QUANTITATIVE ESTIMATION

In the classification of the proteins given on page 643 it will be noticed that the various classes differ from one another in their behaviour towards solvents. Some, such as the albumins, are soluble in pure water; others, such as the globulins, are almost insoluble in pure water, but soluble in aqueous solutions of salts. Another class is distinguished by the fact that its members are insoluble in water or salt solutions, but dissolve in aqueous alcohol, whilst the members of a fourth class are insoluble in all the above solvents, but dissolve in alkaline solutions, from which they can be precipitated by subsequent addition of acids. In addition to the proteins of these classes, a large number are known, belonging chiefly to the group designated as scleroproteins, which form, to a great extent, the supporting and connective tissue of organisms, which are insoluble in all known solvents. In discussing the systematic investigation of protein-containing material, it will be convenient therefore to treat separately the examination of those proteins which can be obtained in solutions, and those proteins which are insoluble (*i. e.* insoluble without decomposition, *e. g.* hydrolysis) in all known solvents.

A. The Systematic Examination of Proteins Which Can Be Obtained in Solution

When the material contains soluble proteins belonging to several classes, the individuals can be separately extracted and separated from one another by treatment of the prepared (fat-free and disintegrated) material by a series of solvents in the following order:

- (i) Pure water, which dissolves the *albumins*.
- (ii) Saline solutions, which dissolve the *globulins*.
- (iii) Aqueous alcohol, which dissolves the *prolamines*.
- (iv) Dilute alkaline solutions, which dissolve the *glutelins*.

The prolamines and glutelins have so far been isolated only from material of vegetable origin. Animal tissues are therefore extracted only by the two first named solvents. Certain plant material contains, however, all the above classes. In addition, plant and vegetable tissues contain in a large number of cases *conjugated proteins*,

especially *nucleoproteins*. These are often extracted by water in the form of a soluble salt, and can be precipitated from solution by the careful addition of acetic acid, either before or after separation of other proteins by coagulation. In certain cases they are extracted by sodium carbonate solution. They contain phosphorus, and some idea as to the amount present can be formed by estimating the phosphorus in organic combination in the solution. The details of their preparation and their properties will be considered later. It must be remembered that these substances and other conjugated proteins can be extracted by water from protein-containing material, some of which is relatively rich in these substances, and their presence in aqueous extracts must not be overlooked.

Notes on the Methods of Manipulation.—Although the processes involved in the examination of protein-containing material appear at first sight very simple, the actual manipulation is often a matter of some considerable difficulty, necessitating a certain amount of experience for the successful accomplishment. The chief difficulty is connected with the filtration of the extracts.

(a) *The Solvents and the Methods of Extraction.*—When water is employed for the extraction, it must be remembered that the material often contains salts. The aqueous extract may in these cases contain, therefore, the globulins. If much water is employed for extraction, the saline solution will be so dilute that relatively little globulin will be dissolved. If the extract is submitted to dialysis in a parchment membrane, until the solution in the dialysing membrane is practically free from salts, the globulins will be precipitated and can then be filtered off; the filtrate will contain the albumins and water-soluble conjugated proteins.

The process of aqueous extraction is often facilitated by agitating the material under examination with the solvent in a shaking machine.

Plant globulins are usually extracted with warm (60°) 10% sodium chloride solution. Sodium salicylate in $N/4$ solution is also a very good solvent, and extracts most plant globulins when cold (Schryver, *Proc. Roy. Soc.*, 1911, 83B, 96). In this case, far smaller quantities of solvent can be used for extraction than when sodium chloride is employed. The amount of solvent to be used in each case will depend on the globulin content, and can be readily ascertained by preliminary experiment with small quantities of material. Five or

six times the weight of sodium chloride solution is usually ample, and when $N/4$ sodium salicylate is used, an equal bulk of the solution will often suffice. (For methods of filtering the pasty material thus obtained, see notes on filtration, below.) The animal globulins are soluble in salt solutions of lower concentration. (See Mellanby, *J. Physiol.*, 1905-6 **33**, 338.)

The alcohol-soluble proteins are practically insoluble in pure alcohol and water. The optimum concentration for the solvent is 73% alcohol. The glutelins can be obtained from the residue of the material after extraction with water, salt solutions, and alcohol, by treatment with 0.5% potassium hydroxide solution. It is advisable to submit the dried residue to this extraction by alkali. The drying is accomplished by treating the residue, after exhaustive extraction with 73% alcohol, with gradually increasing strengths of alcohol, and finally with ether, and removing the last traces *in vacuo*. The material can then be readily powdered.

In all cases, when a quantitative estimation of the various proteins is required, the material should be extracted to exhaustion by the above-mentioned solvents in the order given. The first extract can be made up to a definite volume after the solvent and material under examination have been a sufficiently long time in contact (with, when necessary, mechanical agitation), and the protein dissolved can be estimated in an aliquot part of the extract. To determine whether a particular protein has been entirely removed, the ordinary protein tests (pp. 645 and 648 *et seq.*) can be applied to the successive extracts.

(b) *On the Methods of Filtration.*—The filtration of protein solutions is often a process of considerable difficulty, owing to the slowness with which they pass through ordinary filter paper. Precautions must in all cases be taken to avoid putrefaction during the process. This can be generally avoided by the addition to the solutions of various antiseptics, of which the chief employed are toluene, menthol, thymol and sodium fluoride. (Note: The commercial preparation of this substance is often markedly alkaline.) Antiseptics which act chemically on proteins, such as formaldehyde or mercuric chloride, should be avoided. Even when the danger of putrefaction can be avoided, however, the process of filtration by ordinary methods, especially of large quantities of liquid is so prolonged and tedious as to be impracticable. In working with small quantities of material,

enough solution can generally be obtained by filtration through folded filter papers. When carrying out quantitative investigations, an aliquot portion of the total liquid used for extraction can be filtered through, and used for subsequent work.

Various modifications of the ordinary filtration methods have been suggested when working with protein solutions. Mucilaginous matter can often be separated in a satisfactory manner by filtration through muslin or gauze. Egg-white solutions can be filtered in this manner. Some solutions which will not filter through paper sheets can be filtered through paper pulp. This is made by shredding filter paper and rubbing up the pieces to a thick paste with water, or the solvent employed for extraction (*e. g.*, saline solution) and then throwing the paste on to a Buchner funnel, and drawing off the excess of liquid by means of a filter suction pump. The thickness of the layer convenient for the process can be ascertained by preliminary experiment. This will depend upon the fineness of the suspended particles, and the amount to be filtered. As the filter becomes clogged, the solid particles can be removed by scraping from the surface of the pulp.

In many cases this method of filtration is hardly more satisfactory than the employment of the ordinary filter, and for dealing with these a special process has been devised by Osborne. This consists in adding shredded filter paper to the mixture of the solvent and the material undergoing extraction until an apparently solid mass is obtained. This mass is then subjected to high pressure in a Buchner press, by means of which a pressure as high as 300 kilo. per square cm. can be obtained, and continuing the pressure until most of the added liquid has been squeezed out. The slightly turbid filtrate obtained in this manner can, as a rule, be readily filtered through the ordinary filter, or through paper pulp. It is advisable that the amount of liquid should be small in comparison with the solid, otherwise very large quantities of filter paper are necessary to produce the apparently dry mass. When the quantities are large, as much as possible of the liquid should be separated either by decantation or filtration through a folded filter. Osborne himself has applied it to the residues left on large filters, after the filtration has continued for three or four hours. It is often possible, however, to use quantities of solvent which are small in comparison with the material to be extracted (as in the case mentioned above, where sodium salicylate

solution is employed for the extraction of plant globulins. In this case Osborne's method can be directly applied to the mixture).

In other cases, filtration can be entirely avoided by centrifugalisation. The solid particles then separate at the bottom of the containing vessels, and the supernatant fluid can be pipetted off. To get quite clear supernatant fluids, prolonged centrifugalisation at high speeds may be necessary. In separating moderately large amounts of protein from fine suspensions, in cases where it is not essential to obtain the filtrate entirely free from protein, the Sharples centrifuge is useful. This is a machine constructed on the principle of the cream-separator; the liquid is introduced by means of a needle-valve from a container; the solid collects in an almost dry form in a central hollow cylinder of the machine, while the filtrate passes away.

The Separation of the Proteins from the Solutions in Various Solvents

(i) **The Separation from Aqueous Solution.**—Where the material has been subjected to aqueous extraction, substances which are insoluble in pure water are often extracted owing to the presence of salts. Attention has already been called to this point. This same remark also applies to certain fluids, such as blood serum, which, owing to the presence of salts, contain globulins, which are practically insoluble in pure water. When such substances are present, the aqueous extract or other fluid should be subjected to dialysis until it is practically salt-free. Care must be taken to prevent putrefaction during the process; and disinfectants such as toluene, thymol or chloroform should be added. During the process of dialysis, the globulins separate and can be filtered off. The filtrate will then contain the albumins, nucleoproteins or other water-soluble proteins. Various methods exist for separating these materials. In any case where a preparation free from salts or other simple substances is required, the solution should be submitted to preliminary dialysis.

(a) *The Method of Desiccation.*—The water can be directly evaporated and the proteins obtained as a dry residue. Care must be taken that during this process the temperature does not rise too high, otherwise albumins undergo coagulative change. It is inadvisable that the temperature should exceed 37° . Where the protein

solution is dilute, it can be best treated by first concentrating in an ordinary vacuum distillation apparatus. The concentrated solution should then be evaporated down further in shallow layers in vacuo. Petri dishes, such as are employed in bacteriology, are very convenient for this purpose. The proteins are often obtained in the form of scales, which can be readily scraped off from the bottom of the dish. When the solutions are sufficiently concentrated, or when the quantities of liquid to be manipulated are small, the solution can be evaporated directly in thin layers, without previous evaporation in a distillation apparatus. The final evaporation can be carried out either in a stream of air in an incubator, (37°) or in a desiccator, either at ordinary temperature or at 37° . In any case, evaporation must be sufficiently rapid to prevent putrefaction. If the liquid is placed in shallow dishes in sufficiently thin layers, desiccation will generally follow after leaving for one night at 37° , without forced draught. An apparatus for working with forced draught is described on page 654.

(b) *Method of Precipitation by Organic Solvents*.—The proteins are precipitated from aqueous solution by throwing the latter into 5–10 times the volume of alcohol or acetone. If the precipitate is allowed to remain too long in contact with the solvent, coagulation is apt to take place. A convenient method of precipitation by ice-cold alcohol has already been described (p. 664). By this method, unchanged natural proteins can be obtained.

(c) *Method of Precipitation by Salts*.—This is one of the most important methods for the preparation of proteins—in fact, the only method available for the separation of various water-soluble proteins from one another. The proteins differ from one another in that they are separated from solution by different concentrations of various salts. Thus, the serum globulins are precipitated (from dilute salt-solutions) by saturation with sodium chloride or magnesium sulphate, or by half saturation with ammonium sulphate whereas the serum albumins are not precipitated at all by the two first-named salts and are completely precipitated by ammonium sulphate only when this salt is added in such quantities as to saturate the solution nearly completely. The degree of saturation at which precipitation begins or ends for any particular salt are characteristics for each individual protein; they vary slightly with the concentration of the protein solution, a convenient strength for determining these factors

being about 1%. The method of separation of proteins by the salting-out process was systematically investigated by Denis (*Nouvelles études chimiques*, Paris 1856, and *Memoire sur le Sang*, Paris 1859) and was the subject of many subsequent investigations by Hofmeister and his pupils, and by Kuhne, who applied the process to the separation of the digestion products of the proteins. The property of precipitating proteins by a salt is an additive one, depending upon both the acid and the metal. In actual practice, only a limited number of salts have been employed for the purpose, these being sodium chloride, and the sulphates of sodium, ammonium, magnesium and zinc. Some salts, such as potassium sulphate, are unavailable for the purpose, owing to the fact that they reach their limit of solubility in water before they can precipitate.

The limits of precipitability of a protein by salt solutions may be ascertained in the following way: To 2 c.c. of the protein solution contained in a series of test-tubes are added 8 c.c. of liquid containing varying quantities of water and a saturated solution of the salt under investigation. The lower limit denoting incipient precipitation is observed. The precipitate in the test-tubes containing larger concentrations of the salt is filtered off, and one or two drops of the salt solution are added to the filtrate. As soon as these fail to produce a precipitate, the higher limit of precipitation is reached. If more than one protein is present, a second precipitation will be observed on addition of further quantities of salt after the higher precipitation limit has been reached if the second protein has different precipitation limits to the first. The lower and higher precipitation limits of the second protein can be determined in a series of test-tubes to which salt solution of higher concentrations has been added. In the case of the digestion products of the proteins which will be discussed in detail later (p. 716), several fractions with different precipitation limits have been isolated; the isolation of such products has been one of the most important applications of the "salting-out" method.

In separating proteins by this process, two alternative methods are applicable; the fractions can be separated either by different concentrations of the same salt, or different salts can be employed. Thus in the case of the serum proteins, the globulin fraction can be separated either by saturation with sodium chloride or magnesium sulphate, which do not precipitate the albumin fractions, or by half-saturation with ammonium sulphate; the albumin fraction is com-

pletely precipitated only when the solution is nearly fully saturated by the last-named salt.

It must not be supposed, however, that the fractions obtained by the salting-out method are definite chemical entities. A quantitative separation of different proteins can no more be accomplished by fractional salt precipitation, than can the separation of several liquids with not far removed boiling points by the method of fractional distillation. A precipitate produced by, for example, half-saturation with ammonium sulphate, will contain a certain amount of the protein which is precipitated by full saturation, and *vice-versa*, the precipitation of a substance which for the most part comes down on half-saturation, is not complete. (For an experimental investigation of this subject, see Haslam, *J. Physiol.*, 1905, **32**, 267, and 1907, **36**, 164.) Purer fractions can be obtained by repeated re-solution of the precipitates in water, and re-precipitation at a given volume in a definite degree of saturation with a given salt. For this reason, the salting out method can only be used quantitatively in an empirical way; thus, for example, it is possible to estimate the amount of protein precipitated from a given dilution of serum on half-saturation (globulin fraction) and complete saturation (albumin fraction) with ammonium sulphate, and to determine the changes in the relative amounts of these fractions under different circumstances, such as in samples of serum removed from the same animal in varying conditions of nutrition. In a similar way it is possible empirically to separate digestion products of proteins into fractions; in all cases it must be remembered that the fractions thus obtained are not chemical entities. With pure proteins, on the other hand, the precipitation limits for any salt may be regarded more or less as definite physical constants for any given concentration of the protein.

The method can often be conveniently employed for separating a protein from a large bulk of solution. The precipitate obtained by salting-out can be dissolved in a small amount of water, after washing with salt-solution of the same degree of saturation in which the precipitation was carried out. The solution thus obtained can be freed from salt by dialysis and the protein obtained therefrom by the method of desiccation described above.

(d) *The Separation of Proteins from Solution in a Crystalline Form.* Several proteins can be obtained from solution in a crystalline form. From aqueous solution, egg- and serum-albumins and haemoglobin

can be prepared in the form of crystals, and from saline solutions various globulins (see below, p. 679). It will suffice here to indicate the method for preparing crystalline egg-albumin. (Hopkins and Pinkus, *J. Physiol.*, 1898, **23**, 130.) Egg-white is beaten to a froth (to break up the membranes) with exactly its own bulk of saturated ammonium sulphate solution. After standing overnight, or at least for a few hours, the precipitated protein is filtered off. The filtrate is now measured. 10% acetic acid is then very gradually added from a burette until a definite precipitate is formed, and not a mere opalescence due to liberated gas bubbles. The amount of acid necessary to produce this will vary (chiefly because of the varying loss of ammonia when the liquid has been allowed to stand in open vessels). This point corresponds to the point of incipient acidity to litmus. When this stage is reached, 1 c.c. of the diluted acetic acid for each 100 c.c. of liquid is added, in addition to that already present. The precipitate thus produced is at first amorphous, but on shaking occasionally and allowing it to stand for 24 hours, the full yield of crystals will be obtained. The product can be recrystallised by dissolving it in water and adding half-saturated ammonium sulphate containing acetic acid in the proportion of 1:1,000 till a permanent precipitate forms, and then adding 2 c.c. of the acid ammonium sulphate mixture for each 100 c.c. of the liquid in excess of this. The crystallisation has been found to proceed most satisfactorily when the solution has a pH of 4.8.

Serum albumin can be obtained crystalline in a similar way, if the liquid is made acid with sulphuric acid instead of acetic acid. Crystallisation takes place more readily at somewhat higher temperatures (35–40°) (Inagaki, *Verh. phys. med. Gesellsch. Würzburg*, 1906, **38**, 17).

The relationship of these crystalline proteins to the natural proteins is not accurately known. They differ in certain physical properties.

(ii) **The Separation of Proteins from Saline Solutions.**—When a protein is insoluble in water, but soluble in saline solutions, it can be separated from the latter either by dilution with water or by dialysing away the salt, or by “salting out.” Certain proteins of this class can also be obtained in crystalline form.

When the protein is separated by salt precipitation, it is sometimes possible to employ the same salt as that from which the saline solution is made. Thus, if the globulin is dissolved in ammonium sulphate solution, it can be precipitated by increasing the salt

concentration until the precipitation limit is reached. In other cases, where precipitation in a given saline solution does not take place even when the solution is saturated, another salt must be employed.

Many of the plant globulins can be obtained from the saline solutions in crystalline form, and as an example of the method, Osborne's modification of the earlier methods for preparing crystalline edestin may be quoted. The hemp seeds are first milled and the oil extracted with petroleum spirit, the residue being sieved. 1 kilo. of the powder thus obtained is then treated with 2 litres of 10% sodium chloride solution. To this mixture is then added 800 c.c. of 10% sodium chloride solution to which has been added half the amount of concentrated barium hydroxide solution necessary to produce an alkaline reaction to phenolphthalein. This quantity is determined by estimating the amount of barium hydroxide necessary to produce a pink coloration with an aliquot part of the saline-estelin mixture. The object of the addition of the barium hydroxide is to set free edestin from its combination with acid, as it exists in the saline solution partly in the form of salt. The whole is then thoroughly stirred and thrown on to three or four large folded filter papers. After three hours, about 1,500 c.c. of liquid should filter through. The residue and filters are then mixed with filter paper so as to form an apparently solid mixture, which is then pressed in a Buchner press (see method already described, p. 673). The liquids thus obtained are filtered through a paper-pulp filter (p. 673) and from the slightly opalescent solution thus obtained the edestin can be separated in crystalline form by one of two methods. Either the solution can be placed in large dialysing membranes and the salt dialysed away, when the edestin after 3 or 4 days separates, and can be filtered off on a hardened filter paper and then washed with alcohol and ether and dried over sulphuric acid *in vacuo*, or the opalescent solution is diluted with water heated to 70° until the sodium chloride concentration is reduced to 3%, and then allowed to stand for some hours at 5° when the greater part of the edestin separates. It can be recrystallised by making an 8% solution in a 10% saline solution, warming the solution thus obtained, after filtering it through paper pulp, to 50°, and diluting with twice the volume of water at the same temperature. On cooling this mixture to 5° a very pure edestin is obtained. This should be washed after

filtration with 0.5% saline, then with 50% alcohol till free from chloride, and finally with increasing strengths of alcohol and ether and then dried *in vacuo*. If allowed to dry in the presence of moisture and carbon dioxide, horny masses are obtained which will not dissolve in salt solutions. A more convenient way of drying the edestin, without the formation of horny masses, is by means of acetone (see below, end of next Section).

Edestin may also be extracted with $N/4$ sodium salicylate from which solution it can be precipitated by diluting with 10 times the volume of water (see p. 671). The precipitate thus obtained can be recrystallised from sodium chloride solution by the method described above.

Many other plant proteins can be prepared in a similar way.

(iii) **Separation of Proteins from a Solution in Aqueous Alcohol.**—70% alcohol is a convenient solvent for gliadin and other alcohol soluble proteins. The extract is evaporated *in vacuo* (temperature of the water-bath not to exceed 70°) while the liquid is turbid. If the protein separates out in an oily form, more strong alcohol must be added to bring it into solution. The thick syrup thus obtained is then thrown into 6 to 8 times the volume of ice-cold distilled water to which a small quantity of salt has been added. The protein then separates and is redissolved in strong alcohol, with the addition of water, if necessary, *i. e.*, if sufficient is not present in the original precipitate to make the alcohol sufficiently dilute to dissolve it. The solution is then thrown into cold water and the process of solution and precipitation again repeated, if necessary. By this means the protein is freed from carbohydrates and other non-protein substances. The precipitate is then taken up with alcohol, the extract evaporated, and absolute alcohol added from time to time, so long as the solution remains clear. The evaporation and addition of alcohol is continued until a thick syrupy solution in concentrated alcohol is obtained. This is then thrown in a fine stream into 8 to 10 times the volume of strong alcohol. The protein then separates as a sticky mass, which if kept under absolute alcohol gradually becomes hard and can be powdered. It is disintegrated under alcohol, and rapidly washed, with exclusion of air moisture as far as possible, then with alcohol and ether, and finally dried *in vacuo* over sulphuric acid. If moisture gains access to the mass, it is reconverted into a pasty condition.

For the final granulation of gliadin, dry acetone is a convenient reagent. The syrupy solution of the protein is thrown in a thin stream into several volumes of acetone, whereby the protein is precipitated as a white mass, and is readily powdered under the acetone. It is filtered off, washed with dry acetone, and dried *in vacuo* over sulphuric acid. Thus prepared, gliadin is obtained in the form of a fine powder, which is not hygroscopic.

(iv) **Separation of Proteins from Solutions in Alkalies.**—The proteins which are insoluble in water, salt solutions and alcohol, are also, as far as they have yet been described, plant proteins. The typical example, glutenin, is obtained from wheat meal or gluten after extraction of the alcohol-soluble protein (gliadin), by treatment with 0.5% potassium hydroxide solution. The solution is filtered through pulp, and the proteins precipitated therefrom by neutralising with very dilute acetic acid. It can be purified after separating the last traces of gliadin which it is apt to contain, with 70% alcohol, by redissolving in alkali, and reprecipitation with acid.

B. The Preparation of Proteins Which Cannot Be Obtained in Solution

The general method of preparing the proteins of this class consists in separating all the other constituents of the material under investigation, which can be obtained in solution. The treatment will consequently vary in each individual case, according to the nature of the other constituents. Water will remove the soluble salts and carbohydrates; ether or petroleum spirit the fats and other lipid constituents. Insoluble carbohydrates, such as starch, can be removed by digestion with diastase, by means of which they are converted into soluble carbohydrates, and certain digestible protein constituents can be removed by digesting the material with a proteoclastic enzyme; in this way certain scleroproteins, such as horn, can be prepared, as these are not acted upon by the digestive enzymes. References to the methods of preparing the proteins included under the above heading are given in the table on pages 700a.

The Properties and Methods of Preparation of the Conjugated Proteins

A. The Nucleoproteins.—This important class of proteins is widely distributed in both the animal and vegetable kingdoms and is

distinguished by the fact that the protein is in combination with nucleic acid. These proteins are supposed to form a constituent of the nuclei of cells.

Nucleic Acid.—Although nucleoproteins are so widely distributed, they only occur in the majority of tissues in very small quantities—in fact only very few materials contain nucleoproteins in sufficient amounts for it to be possible to isolate and identify the nucleic acid without employing very large amounts of material. The tissues which contain relatively large quantities of nucleoproteins and which are generally employed for the preparation of nucleic acid are the mature spermatozoa of fish, the thymus gland, testicular substance, and yeast cells. Nucleic acid and nucleoproteins are characterised by the fact that they contain phosphorus in organic combination, and the nucleoproteins are to be distinguished from another class of conjugated proteins, *viz.*, the phosphoproteins, by the fact that the latter substances on treatment with 1% sodium hydroxide solution at 37° undergo hydrolysis and yield their phosphorus in the form of phosphoric acid which can be directly precipitated by ammonium magnesium citrate, whereas the phosphorus remains in organic combination in the case of the nucleoproteins when these substances are submitted to the same treatment. The existence of a nucleoprotein in tissues may be surmised, therefore, when it has been ascertained that the material contains phosphorus in organic combination in a substance insoluble in alcohol (to exclude certain phosphorus-containing lipoids, which are soluble in alcohol) and which is not set free as phosphoric acid when the material is treated with 1% sodium hydroxide solution at 37°. The most certain method, however, of determining whether a nucleoprotein is present consists in directly preparing the nucleic acid itself, but as this is, owing to the small quantities present, only possible in a very limited number of cases, the nucleoprotein should be isolated by the method given below, and the mode of combination of the phosphorus present ascertained by the process already described.

The method generally employed for the isolation of nucleic acid is that originally suggested by Neumann (*Arch. f. Anat. u. Physiol.*, 1899, *Supplementband* 552) who showed that nucleic acid is relatively very stable toward alkalis. This consists in treating the finely minced or otherwise disintegrated material with hot alkaline solutions containing sodium acetate, neutralising the extract thus obtained

with acetic acid, and throwing the product into alcohol. Nucleic acid is thereby precipitated in the form of its sodium salt. In preparing the substance from thymus gland, the tissue is first hardened by boiling with alcohol, after which it can be readily minced in a mincing machine. For 1 kilo. of substance, 2 litres of water, 100 c.c. of 30% sodium hydroxide and 200 grm. of sodium acetate are employed. The filtered extract is neutralised with 150 c.c. of 50% acetic acid, evaporated to 500 c.c. and the solution thus obtained is cooled to 40° and thrown into an equal volume of 96% alcohol. The preparation can be purified by dissolving it in water, adding a small quantity of sodium acetate solution, and throwing the solution into alcohol; without the presence of sodium acetate precipitation does not take place. The free acid can be obtained from the purified sodium salt by throwing a solution of the latter into 3 times the volume of alcohol, to which 2 c.c. of concentrated hydrochloric acid has been added to every 100 c.c. In the presence of mineral acids, nucleic acid is unstable and undergoes hydrolysis.

A method for preparing nucleic acid from yeast is described by Clarke and Schryver (*Biochem. J.*, 1917, **11**, 319). Yeast is dried by treating it with a large excess of alcohol, first in the cold, and then on a water-bath, after which the solid is filtered off and dried in a current of air at 37°. The dry material is extracted with 10 times its weight of 10% sodium chloride solution for some days at 60–80°, and the clear filtered extract is treated with one tenth its volume of hydrochloric acid (1:1), with vigorous stirring. After standing for 2 hours, the cake of nucleic acid is removed and washed with 50% alcohol. The crude material is purified by the method of Osborne and Harris (*Z. physiol. Chem.*, 1902, **36**, 85). It is dissolved in 25 times its weight of 10% sodium acetate solution, warmed on a water bath, and filtered. From the clear filtrate, the nucleic acid is precipitated by adding one-fifth the volume of alcohol, and excess of hydrochloric acid. The white precipitate is washed free from chloride with 50% alcohol, then with 95% alcohol, and ether, and dried *in vacuo*.

Nucleic acids derived from plant and animal sources are similar in composition, but differ in important particulars. On hydrolysis by acids they yield: (i) phosphoric acid, (ii) a sugar or sugar derivative (iii) bases of the purine and pyrimidine types. The sugar obtained from plant nucleic acid is a pentose, *d*-ribose; animal nucleic acid

contains a hexose which is converted during hydrolysis into formic acid and laevulinic acid. The bases obtained on hydrolysis are (from animal nucleic acid) guanine, adenine, cytosine and thymine; among those from plant nucleic acid, thymine is replaced by uracil.

Nucleic acids consist of a combination of four nucleotides, each nucleotide being a complex containing phosphoric acid, a carbohydrate, and one of the above-mentioned bases. The nucleotides can themselves, by suitable treatment, be converted into nucleosides—substances composed of one of the bases in combination with a carbohydrate molecule. For a more complete account of the chemistry of the nucleic acids, the reader is referred to the monograph on "*Nucleic Acids*" (Jones; Monographs on Biochemistry, London).

The Nucleoproteins.—The nucleoproteins are now regarded as combinations of nucleic acid with certain basic proteins. This view is borne out by the observation that pure nucleic acid, prepared from thymus gland, combines with varying proportions of some proteins, behaving as a polybasic acid.

By extraction of nucleoprotein-containing tissue with cold solvents, the so-called α -nucleoproteins are obtained; if these substances are heated, or hot solvents employed, a certain amount of protein is coagulated, and there remains in solution the so-called β -nucleoprotein. If nucleoproteins are submitted to hydrolysis by proteoclastic enzymes, part of the protein undergoes degradation; the nucleic acids are not attacked by the ordinary digestive enzymes.

In a few cases, definite proteins have been isolated from the nucleoproteins, substances of the strongly basic protamine or histone types being obtained. The materials which have been chiefly employed for the preparation of histones are the blood corpuscles, of birds and snakes, the thymus gland, and the testicles of the fish *gadus* and *lota*. The protamines have been prepared from the matured testicles of salmon, herring and other fish. The general method of preparation consists in extracting the material with acid, and precipitating the protein as a salt of the acid by alcohol. They can also be precipitated in *neutral* solution with the ordinary alkaloidal reagents. The protamines are generally purified by precipitation as picrates.

The general method of preparing nucleoproteins consists in extracting the tissues with either water or dilute alkali, and precipitating the nucleoprotein from the solution with dilute acid. The

protein can be purified by repeated solution in weak alkali and precipitation with weak acids. It is often advisable to prepare the material before extraction by treating it with alcohol (to extract lipoids) and then with ether, and grinding to a fine powder. In the majority of cases, the yield obtained is only very small.

B. The Phosphoproteins.—These substances are distinguished from others by the fact that on treatment with 1% sodium hydroxide solution at 37° for 24–48 hours the whole of the phosphorus is set free from organic combination. These substances are not widely distributed, and occur chiefly in milk, egg-yolk, and the ova of fishes, that is, in the substances which constitute the food-stuffs of the embryo bird and fish and the young mammal. There is also a small quantity present in the pancreas. The two proteins of this class which have been chiefly investigated are caseinogen from milk and vitellin from egg-yolk. The substances are fairly strongly acidic (see below, p. 699). Caseinogen can be prepared by the following modification of the original method of Hammarsten: 2 litres of separated milk are diluted with 8 litres of water, and 10 c.c. of glacial acetic acid are then added. The caseinogen is thereby precipitated, and the supernatant liquid is decanted off. The precipitate is washed with 2–4 litres of slightly acid water and then squeezed in a cloth. It is then rubbed up to a thick paste with 1–2% ammonia solution, which is afterwards diluted with 1,000–1,500 c.c. of ammonia of the same strength. The fat separates at the surface of the solution as the caseinogen is dissolved. The liquid is separated from the fat, and then 10–12 c.c. of glacial acetic acid are added. The caseinogen is thereby precipitated and is purified by redissolving in ammonia and reprecipitating by acid. The process can be repeated as often as necessary. (See section on *Milk Proteins*.)

The following method, due to Van Slyke and Baker (*J. Biol. Chem.*, 1918, **35**, 127), is convenient and gives a very pure product. Fresh milk is used, and *N*/1 lactic acid, or a mixture of equal parts of *N*/1 acetic acid and *N*/1 hydrochloric acid, is run in very slowly, being introduced in a very thin stream under the surface of the milk, and close to a rapidly-rotating stirrer. The rate of addition of the acid should be about 30 c.c. in 40 minutes, and the stirrer should be driven at 3,000 r.p.m. After the addition of 60 c.c. of acid per 1,000 c.c. of milk, the addition of acid is stopped, but stirring is continued for

3 hours at a reduced rate (1,000 r.p.m.). More acid is then run in, in the same manner, until a test portion of the liquid, mixed with an equal amount of water, gives an immediate sediment on rotating in a centrifuge. (Usually 80-90 c.c. of acid per litre of milk are required in all.) The whole liquid, after stirring slowly for 2 hours more, is centrifuged, the precipitate being well washed with distilled water. Northrop (*J. Gen. Physiol.* 1923, 5, 749) recommends further purification as follows: 100 grm. of the above product are suspended in 1,000 c.c. of water, and hydrochloric acid is then added to bring the acidity of the liquid to a pH of 2.5-3.0. The resultant cloudy solution is filtered till quite clear, if necessary repeatedly. The pH of the filtrate is then adjusted to 4.7 by the addition of sodium hydroxide, and the precipitate of casein is collected, washed several times with distilled water, and dried with acetone. The product gives an almost water-clear solution in either acid or alkali.

Vitellin can be prepared in the following manner (Levene and Alsberg, *Z. physiol. Chem.*, 1901, 31, 543): Egg-yolks are mixed with an equal volume of 10% sodium chloride solution and the mixture is extracted three times with ether, the mixture being allowed to stand each time for 24 hours in contact with ether. A liquid is then obtained which can be readily filtered, and from which the pigment can be extracted with ether. It is diluted with 20 times the volume of water. The precipitate thus formed is allowed to settle, then redissolved in sodium chloride solution, which is extracted afterwards with ether, and the vitellin is reprecipitated by dilution with water. The precipitate thus obtained is extracted first with alcohol, and afterwards with ether, until nothing more is extracted by this solvent.

C. The Glycoproteins.—The chemical knowledge of substances of this group is still very small. The members of the class are characterised generally by the fact that their solutions in alkalis are mucilaginous and yield on mild hydrolysis substances which reduce Fehling's solution. The cause of this reduction lies in the liberation, on hydrolysis, of glucosamine or galactosamine. The principal members of this class are the mucins and the cartilaginous substances, which latter substances, from the fact that they yield chondroitin sulphuric acid on hydrolysis, are sometimes called the chondroproteins. Of the mucins, some are precipitated by acetic acid and are insoluble in excess of the acid (true mucins); others are soluble in

excess of the acid (mucoids) whereas others again are not precipitable by acetic acid (pseudomucins). According to their properties they are precipitated from solutions by either acetic acid or alcohol, and their separation from other substances is largely a matter of mechanical treatment; they are precipitated in the form of sticky strands of substance which can be collected on a glass rod. The homogeneity of many of the substances is doubtful.

METHODS OF IDENTIFICATION OF THE INDIVIDUAL PROTEINS¹

A. Physical Constants

The establishment of physical constants of the individual proteins is a matter of considerable difficulty, and is beset with many pitfalls. In the first place, attention has already repeatedly been called to the difficulty of quantitatively separating the proteins from one another; in the second place, these colloidal substances possess the property of absorbing salts and other simple substances from solution, and these absorbed substances profoundly affect the physical constants; in the third place, the proteins are substances both basic and acidic in character, and capable of forming salts both with acids and bases. As the molecular weights of proteins are high, the presence of minute quantities of acids and bases may also profoundly affect the physical constants. It is only necessary to instance here the great differences in the optical rotation of any substance and its salt; leucine, *e. g.*, has an optical rotation $[\alpha]_D^{20} = -10^{\circ}.42$, whereas that of its hydrochloride is $[\alpha]_D^{20} = +15^{\circ}.33$ in 20% hydrochloric acid. It may be easily understood from this example that a minute quantity of acid in a protein solution may easily cause a great change in any physical constant. Furthermore, there is a great difficulty in determining whether any inorganic substance is in combination with a protein or whether it is merely physically adsorbed, and on this subject an extensive literature exists. From these introductory remarks, it will be obvious that a physical constant of a protein cannot be determined without submitting the substance to an extremely careful purification, such as prolonged dialysis or by other available methods. Few physical constants, therefore, can be employed in the ordinary routine of fixing the identity of a given protein; for this purpose certain chemical methods, described in detail later, are

¹ For the meaning of the asterisk at the commencement of certain sections, see p. 688.

simpler and give far more reliable results. It will suffice therefore, in the sequel, to refer in most cases only very shortly to the physical methods of identifying proteins. Those processes which seem applicable to a routine examination are indicated by an asterisk.

(i) *Solubilities of Proteins in Various Solvents*.—The differences of the solubilities of proteins in various solvents has already been repeatedly referred to in the discussions on the methods of preparation. When they are soluble in water or dilute alcohol, thick syrupy solutions can be obtained, when small quantities of the solvent are employed. The proteins are soluble in these solvents in all proportions. The solubilities of the globulins in salt solutions, on the other hand, vary very considerably, and the degree of solubility can, in many cases be accurately measured. (See Hardy, *J. Physiol.* 1905, **33**, 254; Mellanby, *J. Physiol.*, 1905, **33**, 338; Osborne and Harris, *Amer. J. Physiol.*, 1905, **14**, 151; Schryver, *Proc. Roy. Soc.*, 1910, **83 B**, 96.)

*(ii) *Precipitability of Proteins by Salts*.—The method of determining these constants has already been given. The precipitation limits are important in the case of the digestion products of the proteins, which are discussed in a separate article. (See p. 716.) The determinations have been made with various salts on animal proteins (see p. 675, and Lewith, *Arch. Exp. Path. Pharm.*, 1887, **24**, 1), and with ammonium sulphate in the case of various vegetable proteins (Osborne and Harris *J. Amer. Chem. Soc.*, 1903, **25**, 848).

(iii) *Coagulation Temperature and Clotting Point of Proteins*.—The coagulation temperature will depend upon the reaction of the medium, the presence of salts or other substances, and upon the rate at which the mixture is heated. Hitherto the coagulation point has been regarded as a constant, but, for the reasons given above, this is no longer possible. The subject requires, therefore, no further discussion in this place.

(iv) *Optical Rotation of Proteins*.—With the exception of haemoglobin, for which $[\alpha]_D = +10.4$ (Gamgee and Croft Hill, *Ber.*, 1903, **36**, 913) and some nucleoproteins, the proteins are laevo-rotatory. The number of reliable estimations of optical activity is small (see Osborne and Harris, *J. Amer. Chem. Soc.*, 1903, **25**, 842).

Dakin (*J. Biol. Chem.*, 1912, **13**, 357) noted that in alkaline solutions ($N/2$ sodium hydroxide) the specific rotation of a protein drops considerably; this change he termed "racemisation." It

was shown by Woodman (*Biochem. J.*, 1921, **15**, 187) that the Racemisation Curve, obtained by plotting the specific rotation against time, was, under standard conditions, characteristic for any given protein, and could be used as a test of identity. It was further shown that after racemisation was complete, acid hydrolysis yielded a mixture of optically active and inactive amino-acids; the nature of those amino-acids which were thus obtained in the optically inactive form has also been used to distinguish between similar proteins. (Dakin and Dale, *Biochem. J.*, 1919, **13**, 248.)

(v) *The Molecular Weight of Proteins.*—The molecular weight of some proteins can be determined from measurements of osmotic pressure in solution; the presence of small amounts of salts or other electrolytes greatly influences the results obtained. The subject of molecular weight of proteins still requires a considerable amount of investigation. For a review of the position in this respect, see Cohn, Hendry and Prentiss, *J. Biol. Chem.*, 1925, **63**, 721.

(vi) *Refractive Index of Protein Solutions.*—The refractive index of protein solutions may be expressed by the formula $n - n_1 = ac$, where n = refractive index of the solution, n_1 = refractive index of the solvent, c = concentration, and a = constant.

Protein	a
Ovomucoid	0.00160
Ovovitellin	0.00130
Caseinogen	0.00152

(Brailsford Robertson, *J. Biol. Chem.*, 1909-1910, **7**, 359.)

*(vii) *The Iso-electric Point of Proteins.*—As the proteins are built up by the conjugation of mono-amino- and diamino-mono-carboxylic acids and dicarboxylic acids, they can contain, according to the groups from which they are formed, varying numbers of free amino or carboxylic acid groups, and consequently combine with varying quantities of acids or bases to form salts. The simultaneous presence of both these groups tends to confer on the majority of the proteins an amphoteric character, so that they act neither as strong acids nor strong bases. In only a few cases is the acid or basic character therefore strongly marked, and the acid or basic functions can be directly ascertained by simple titration with bases and acids (see p. 699). Furthermore, owing to the high molecular weights, solutions containing quite large quantities of protein, *e. g.*, 5% solutions are, technically, dilute, and except in the presence of

excess of acid or base the protein salt will undergo hydrolytic dissociation. Owing to these facts there is often a great difficulty in preparing proteins sufficiently pure for determination of the physical constants, a circumstance to which attention has been drawn repeatedly in the foregoing pages. It will be obvious from these remarks that the acidic or basic functions cannot be readily ascertained by direct titration of the protein solutions by solutions of acids and bases in the presence of indicators. It is possible, however, to determine readily and with a fair degree of accuracy, the so-called "iso-electric point" of a protein, this being a measure of the relative amounts of hydrogen and hydroxyl ions produced in solution when a pure protein is dissolved in pure water. The relative amounts of these ions thus produced will depend upon the numbers of free amino and carboxylic acid groups present in the protein, and the iso-electric point will therefore be characteristic for any given protein. The method of electrometric titration usually employed for the determination of the iso-electric point consists in titrating, over a range of hydrogen ion concentration values, a known amount of standard acid, containing the protein in solution, with standard alkali. The alkali is run into the acid in portions usually of 1 c.c., and the hydrogen ion concentration of the liquid is measured after each addition of alkali (for experimental details of the measurement of hydrogen ion concentration by the electric potential method, see text books on practical physical chemistry). The curve obtained by plotting hydrogen ion concentration (usually expressed as the logarithmic function, pH) against the c.c.s. of alkali added, will show a flexure in the neighbourhood of the iso-electric point of the protein under examination; it is further possible to deduce from such titration curves the acidic and basic dissociation constants of the protein (see Harris, *Proc. Roy. Soc.*, 1924, *B*, **95**, 440, 500 and subsequent papers, for details of the method, etc.).

Two other methods have been employed for the determination of the iso-electric point. It is known that agglutination of a denatured protein takes place most readily when the protein is suspended in an iso-electric solution, and that the iso-electric point of a protein corresponds to the point of minimum solubility (*i. e.*, maximum turbidity). This fact is the basis of a method used by Michaelis for the determination of the iso-electric points of a number of proteins (see *Biochem. Z.*, 1910, **24**, 79; 1910, **27**, 38; 1910, **28**, 193, and sub-

sequent papers in the same journal by Michaelis and his collaborators).

Furthermore, when a protein is suspended in an electric field, it moves neither to the cathode nor the anode when in an iso-electric solution. Thus by determining the hydrogen ion concentration of the medium in which the protein is electrically neutral, its iso-electric point can be ascertained. This is known as the method of electro-cataphoresis. (See Michaelis, *Biochem. Z.*, 1909, 16, 81.)

Below is a table showing the iso-electric points and ionisation constants of certain proteins, as given by the methods outlined above.

Protein	Iso-electric point (pH)	Ionisation constants		Temp.
		k_a	k_b	
Serum albumin.....	4.7	4×10^{-7}	1.6×10^{-10} to 1.6×10^{-11}	at 18°
Serum globulin.....	5.52	1×10^{-5}	2×10^{-10} to 2×10^{-11}	25°
Egg albumin.....	4.8	2.6×10^{-7}	1.2×10^{-11}	25°
Gelatin.....	4.7			
Edestin.....	5.5 to 6.0	2.3×10^{-8}	3.2×10^{-11}	25°
Fibrin.....	6.8			

Iso-electric points have also been determined in the case of some proteins by ascertaining the hydrogen ion concentration at which minimum swelling is observed, and at which minimum absorption of ultra-violet light occurs.

B. Chemical Constants

Several chemical constants have been suggested for the identification of individual proteins, some of which, such as the determination of the acidic or basic functions, are only applicable to materials which have been submitted to very careful purification. In other cases such careful preparation of the material is not necessary, and in these instances the determinations of the chemical constants may be employed as routine methods for fixing the identity of any given protein; and the methods employed for the purpose described in some detail below (paragraphs marked with an asterisk). Of these, that principally in use is the determination of the so-called "Hausmann numbers;" the estimation of the sulphur-content of a protein is occasionally of value.

(i) *The Empirical Composition of Proteins and Their Sulphur Content.—The carbon, hydrogen nitrogen and oxygen content in proteins do not, as a rule, vary within very wide limits, and the numbers obtained by as ordinary combustion are not particularly characteristic for the individual proteins. In the case of nitrogen, it must be remarked, there are occasionally wide variations, and certain basic proteins, belonging to the histones and protamines contain relatively large quantities of this element, but in the vast majority of cases, the variations from a mean are too small to afford any numbers that may serve to characterise a protein. This is not, however, the case with the element sulphur, as the amount of cystine yielded by hydrolysis varies within wide limits, as the following table, giving the sulphur content of some characteristic proteins, shows.

Protein	Sulphur Content, %
Globin (from haemoglobin)	0.42
Serum albumin	1.90
Egg albumin	1.62
Blood globulin	1.11
Egg globulin	0.12
Edestin (hempseed)	0.91
Zein	0.60
Gliadin	1.14
Glutenin	1.08
Caseinogen	0.76
Fibrinogen	1.25
Gelatin (commercial)	0.7
Hair	5.0
Horn	3.20

As the sulphur is readily estimated with a considerable degree of accuracy and as the variations in the amount are great in different proteins, the determination of the sulphur content affords a valuable chemical constant for fixing the identity of proteins. Some proteins also contain phosphorus (phosphoproteins and nucleoproteins). If present its amount should be determined.

The most convenient and rapid method for the estimation of sulphur is that of Benedict, as modified by Wolf and Osterberg (*Biochem. Z.*, 1910, 29, 429). According to this method, the organic material is first oxidised with fuming nitric acid until the solid matter has all passed into solution and nitrous fumes are no longer given off, and the oxidation is finally completed by heating with Benedict's reagent, which is made by dissolving 200 grm. of copper nitrate and 50 grm. of potassium chlorate in 1,000 c.c. of water (Denis modifies the oxidising

solution to contain 25 grm. copper nitrate, 25 grm. of sodium chloride and 10 grm. of ammonium nitrate in 100 c.c. water; 5–10 c.c. of this reagent are usually employed for oxidation). The mixture of the solution of the protein substance in nitric acid with the copper nitrate reagent is first evaporated to dryness and then heated over a free flame to redness for 15 minutes. The sulphur is thereby oxidised to sulphate which is estimated as the barium salt in the usual way (gravimetrically).

Phosphorus is best estimated by Neumann's method (*Z. physiol. Chem.*, 1902, 37, 115), as modified by Plimmer and Bayliss (*J. Physiol.*, 1906, 33, 441). The estimation of sulphur and phosphorus in the same sample can be carried out by the method of Wolf and Osterberg (*loc. cit.*).

(ii) *Estimation of Hydrolysis Products.* *(a) The Determination of the "Hausmann Numbers."—The chemical factors which are most characteristic of the individual proteins are the quantities of the various amino-acids which are yielded by hydrolysis, the methods for estimating which have been discussed in some detail in the earlier pages of this article. It has there been repeatedly stated that the methods are complex and large quantities of material are necessary for investigation; although the separate estimation of the diamino-acids is often feasible (p. 638, *et seq.*). A series of very simple chemical constants which are dependent on the hydrolysis products are the so-called "Hausmann numbers." Although much complicated manipulation is necessary to separate the individual amino-acids, the diamino-acids as a group are, owing to their more basic character, readily separated from the mono-amino acids by the fact that they are precipitable by alkaloidal reagents. Furthermore, most proteins yield on hydrolysis certain quantities of ammonia, which, in the case of some vegetable proteins, are relatively large ("amide"-nitrogen). In addition, a small amount of nitrogen is contained in the insoluble pigmented substance which is separated during hydrolysis and known as "humin" nitrogen. The distribution of the nitrogen among these groups of hydrolysis products is expressed as percentages of the total nitrogen contained in the proteins, and the figures thus obtained are known as the Hausmann numbers. The method of determining these is as follows:

About 1 grm. of protein is boiled with 20% hydrochloric acid, usually for 16–20 hours, until the solution no longer gives the

biuret reaction. It is then evaporated at 40° to 2 or 3 c.c., and the residue is transferred to a flask with about 350 c.c. of water, and cream of magnesia is added in slight excess. The latter must be freed from every trace of ammonia by boiling before addition to the hydrolysis products. The mixture is then freed from the ammonia formed by hydrolysis by heating *in vacuo* to 40° , and passing the distillate into a known excess of *N*/10 sulphuric acid. The amount of this acid neutralised indicates the amount of ammonia formed by hydrolysis. (Termed "amide-nitrogen.") The residue left in the flask is then filtered through nitrogen-free paper, which is then thoroughly washed with water, the washings being added to the original filtrate. The nitrogen ("humin-nitrogen")¹ in the dark-coloured mass left on the filter is then estimated by Kjeldahl's method. The filtrate and washings are then concentrated to 100 c.c., cooled to 20° , and 5 grm. of sulphuric acid are then added. To this mixture are added 30 c.c. of a solution made by dissolving 20 grm. of phosphotungstic acid and 5 grm. of concentrated sulphuric acid in 100 c.c. of water.² The precipitate thus formed is filtered off after standing for 24 hours, and is then washed with a solution containing 5 grm. of sulphuric acid and 2.5 grm. of phosphotungstic acid in 100 c.c. of water. The washing is effected by rinsing the precipitate from the filter into a beaker and returning to the paper three successive times, each portion of the wash being allowed to run out completely before the next is applied. About 200 c.c. of washings are usually obtained. The nitrogen in the precipitate (diamino-acid or "basic nitrogen") is estimated by transferring the precipitate and filter paper to a Kjeldahl flask and digesting with 35 c.c. of concentrated sulphuric acid for 7-8 hours. Crystals of potassium permanganate are occasionally added during this incineration to promote the oxidation processes. Care must be taken to avoid "bumping" during the process. The Kjeldahl estimation is completed in the usual way.

After removal of the precipitate of phosphotungstates, the filtrate is examined, after neutralisation and concentration to a convenient bulk, for mono-amino nitrogen (by the Van Slyke method), and, hence, the non-amino nitrogen may be estimated (by difference). The non-amino nitrogen is due to the presence of proline, oxyproline, peptides etc.

¹ Sometimes called "melanin-nitrogen."

² See note on p. 628.

The reliability of the figures obtained in the determination of the Hausmann numbers has repeatedly been criticised, particularly with regard to the values obtained for "basic nitrogen." The following recommendations are made by Knaggs (*Biochem. J.*, 1923, **17**, 490), who states that concordant results are obtained by adopting the following precautions:

(i) The protein should be hydrolysed by introducing it directly into boiling acid; it should not be allowed to stand with cold acid before hydrolysis (cf. p. 627).

(ii) The solution after distillation of the ammonia (*i. e.*, after determination of the amide and humin nitrogen) should not be allowed to stand for any length of time prior to the addition of the phosphotungstic acid reagent. If the liquid has been standing with sulphuric acid, it should be boiled for a few minutes, cooled, and immediately precipitated with 20% phosphotungstic acid. If these precautions are observed, the precipitate obtained is granular, settles quickly, and is easily filtered and washed.

The question of the precipitation of the "basic" fraction is discussed further by Plimmer and Rosedale (*Biochem. J.*, 1925, **19**, 1005), Gortner and Sandstrom (*J. Amer. Chem. Soc.*, 1925, **47**, 1663) and Kernot and Knaggs (*Biochem. J.*, 1928, **22**, 528).

Recently a micro-method for the determination of the Hausmann numbers has been worked out by Thimann (*Biochem. J.*, 1926, **20**, 1190). By this method the determinations can be carried out with a sample of substance containing only some 15 mg. of nitrogen, *i. e.*, 90-100 mg. of dry protein; this amount allows for duplicate estimations.

The protein is dropped into 30 c.c. of boiling 20% hydrochloric acid contained in a 100 c.c. Kjeldahl flask. The mixture is boiled under a reflux condenser for 18 hours, a hang-in glass condenser being used, sufficiently large to fit closely into the neck of the Kjeldahl flask. After cooling, 2 portions of 10 c.c. each of the hydrolysate are pipetted into hard glass tubes (200 × 25 mm.) and evaporated to dryness *in vacuo* at 40°, precautions being taken against loss by frothing or "bumping." The residue in each case is dissolved in 2 c.c. of 10% sodium hydroxide solution, and sulphuric acid is added until the liquid is just acid. The liquid is transferred to the distillation flask of a micro-Kjeldahl apparatus (see p. 661) and 2% sodium hydroxide solution is added until the liquid is just alkaline to

phenolphthalein. The ammonia is then distilled off in steam into 5 c.c. of *N*/70 sulphuric acid, as in the ordinary micro-Kjeldahl process¹ (*q. v.*). The excess of acid is titrated at once with *N*/70 alkali, the "amide-nitrogen" being thereby calculated.

The residual liquid, after removal of the ammonia, is made acid with 1 drop of concentrated sulphuric acid, and the dark precipitate is removed on the centrifuge, the nitrogen present therein being determined ("humin" nitrogen).

Pure sulphuric acid (0.3 c.c.) is now added to the clear filtrate, the liquid is evaporated down as before, and made up to a volume of 10 c.c. in a marked test-tube. The liquid is boiled (if it has been standing with acid, it should be heated in an autoclave at 130° for 3 hours), cooled in ice, and 3 c.c. of phosphotungstic acid reagent are added, drop by drop, to the cold liquid. After standing in ice overnight the precipitate is centrifuged off, a 15 c.c. urine tube being used, and is washed 4 to 5 times, with a total of 10 c.c. of phosphotungstic acid wash-liquid (see above, p. 694), the hard lump of solid in the tube being broken up with a glass rod after each washing (on the centrifuge). Finally, the solid is dissolved in 2 c.c. of 10% sodium hydroxide, and the nitrogen estimated by the Kjeldahl method ("basic" nitrogen).

The combined filtrates and washings from the phosphotungstates are made up to a volume of 50 c.c., and the nitrogen ("amino" plus "non-amino" nitrogen) is determined in two portions of 10 c.c. each. Total nitrogen is determined in portions (2 c.c.) of the original hydrolysate. The entire analysis takes about three days.

A few typical Hausmann numbers are given in the following table.

Protein	N, %	Amide	Humin	Basic	Mono-amino + non-amino
Egg albumin.....	15.51	8.64	1.87	21.27	68.13
Caseinogen.....	15.62	10.43	3.43	19.61	64.55
Glutenin.....	17.49	18.86	1.08	11.72	68.31
Edestin.....	18.64	10.08	0.64	31.70	57.83
Edestin (micro).....		12.10	0.30	28.80	58.70
Gelatin.....		2.20	0.10	25.50	62.50
Gelatin (micro).....		2.60	0.00	17.60	79.60
Gliadin.....	17.66	23.78	0.79	5.54	70.27
Gliadin (micro).....		26.30	0.10	5.40	68.30

¹ This method of distillation is satisfactory provided that cystine is not present in appreciable amounts. In the presence of cystine, the ammonia is aspirated off in a brisk current of air; the liquid is kept at a temperature of 40°, and the aspiration is continued for 2 hours (naturally, the air must be free from ammonia).

An extension of the method, whereby the individual diamino-acids can be estimated, is due to Van Slyke. The diamino-acids, together with some of the cystine, are precipitated together as phosphotungstates (see above), and their further differentiation depends on the following facts: (a) that when the protein hydrolysis products are treated with nitrous acid, only the nitrogen contained in the form of amino groups (NH_2) reacts with the acid with evolution of nitrogen gas. If reference is made to the formula of the diamino-acids, it will be evident that arginine contains three-fourths of its nitrogen in a form which will not react with nitrous acid, whereas histidine contains two thirds in this form; lysine, on the other hand, contains all its nitrogen in the form of amino-groups. (b) Arginine, on hydrolysis with potassium hydroxide, yields ornithine and urea, and the latter decomposes further into ammonia and carbon dioxide:



Under the correct conditions of experiment, arginine is the only one of the bases, precipitated by phosphotungstic acid, which evolves ammonia on boiling with alkali, half its nitrogen being given off in this form. From this reaction, the amount of arginine in the phosphotungstic acid precipitate can be readily ascertained.

If now the total nitrogen is known, and the amount of nitrogen present in the amino-form (as estimated by treatment with nitrous acid); then if D = non-amino nitrogen (*i. e.*, the difference between total and amino-nitrogen) in the phosphotungstic acid precipitate, the amount of histidine nitrogen can be calculated from the formula:

$$\text{Histidine N} = \frac{3}{4}(D - \frac{3}{4} \text{ Arginine nitrogen}).$$

The lysine nitrogen = total nitrogen - (Arginine + Cystine¹ + Histidine) nitrogen.

For practical details, the reader is referred to Van Slyke's paper (*J. Biol. Chem.*, 1911, 9, 185). The method of estimating amino-nitrogen by means of nitrous acid is described on page 719 of this volume.

The main sources of error in the method lie: (i) in the determination of cystine, since it is now known that the whole of the sulphur in the molecule is not due to cystine, and (ii) in the determination

¹ Cystine is in part precipitated by phosphotungstic acid; the amount present is estimated by determination of the sulphur present in the mixture of free bases.

of the lysine, the figure for which includes the errors arising from several other determinations, as well as the nitrogen which should be ascribed to hydroxylysine (if present).

(b) **Estimation of Certain Individual Hydrolysis Products.**—

(i) *Tyrosine*.—(Weiss-Millon method; see Weiss, *Biochem. Z.*, 1919, **97**, 170.) In this method the colour produced by the action of a modified Millon's reagent on tyrosine is matched against that given when using a standard tyrosine solution, and the relative concentration deduced therefrom. The Millon's reagent used consists of a 10% solution of mercuric sulphate in 5% sulphuric acid, to which is added, immediately before use, a little sodium nitrite. The standard tyrosine solution contains 0.1 gm. of the pure substance dissolved in 95 c.c. of water, with the addition of 5 gm. of pure sodium hydroxide; a little chloroform is added, and the mixture is kept in a brown glass bottle. From this stock liquid solutions are made up containing 1 part of tyrosine in 10,000 and 50,000 parts, respectively, of water. 3 c.c. of the tyrosine solution are mixed in a test-tube with 2 c.c. of the Millon reagent, and 3 drops of 0.5% sodium nitrite solution. The mixture is heated gently to boiling, and left to stand for 5 minutes, by which time the maximum colour develops, and persists for 30 minutes. The tyrosine solution under examination should be diluted to give a colour comparable with that given by the standard 1 in 10,000 or 1 in 50,000 solution.

(ii) *Tryptophane*.—A method for the colorimetric estimation of this amino-acid is due to May and Rose (*J. Biol. Chem.*, 1922, **54**, 213), who find that on heating a protein in the presence of hydrochloric acid and *p*-dimethylaminobenzaldehyde, a blue colour develops, proportional in intensity to the amount of tryptophane present in the protein. The reagent consists of a 5% solution of the aldehyde in 10% sulphuric acid. 1 c.c. of this reagent gives a maximum colour with 0.1 gm. of the protein in which tryptophane is present. It is best to hydrolyse the protein in the presence of the reagent, when the condensation of the tryptophane takes place immediately, without loss by decomposition. 100 c.c. of hydrochloric acid (1 acid: 1 water) and 1 c.c. of the reagent are mixed together, and weighed amounts (0.05 to 0.1 gm.) of the dry protein are added, the mixture being warmed to 35° for 24 hours. This procedure gives clear blue solutions. The colour produced is compared with that produced by a similar amount of pure caseinogen,

which is assumed to contain 1.5% of tryptophane. (See also Boyd, *Biochem. J.*, 1929, **23**, 78.)

(iii) *Histidine*.—Koessler and Hanke describe a method for estimating histidine colorimetrically, by means of the well-known reaction between this amino-acid and *p*-diazobenzenesulphonic acid. Tyrosine and certain other amino-acids must be absent (*J. Biol. Chem.*, 1919, **39**, 497; also Lautenschlager, *Zeitsch. physiol. Chem.*, 1918, **102**, 226).

(iv) *Determinations of the Acid and Basic Functions of a Protein*.—As has already been pointed out (p. 689) the proteins, by reason of their structure, have amphoteric properties, and act neither as strong acids nor as strong bases. In only a few cases is the acid or basic character strongly marked, when the acid or basic functions can be determined by direct titration with an appropriate base or acid. Caseinogen can be titrated with alkalis (Lacquer and Sackur, *Beitr. Chem. Physiol. Path.*, 1902, **3**, 193). The protamines, furthermore, are strong bases. Only in rare cases, therefore, can a protein solution be titrated directly with acid or alkali in the ordinary manner. As has been pointed out above, however, advantage is taken of the weakly acid or basic character of the proteins for their characterisation by means of their iso-electric points (*q. v.*). Methods of determining the iso-electric point of a protein are referred to on p. 690.

(v) *Estimation of the Reactive Amino and Carboxyl Groups*.—The reactive amino-groups in a protein can be estimated by ascertaining the amount of nitrogen set free when the protein is treated with nitrous acid. The estimation presents some difficulty in the majority of proteins, when the amount of reactive amino-nitrogen is low. As, however, the number of reactive amino-groups rises rapidly during the hydrolysis of a protein, the method is a useful one for following the course of a protein degradation, and is described in detail in dealing with the digestion products of the proteins (p. 719).

Similarly, the reactive carboxylic acid groups can be estimated by titration in the presence of formalin. This method, again, is more useful when applied to the study of the degradation of proteins, and is described in greater detail in the article dealing with the digestion of proteins (p. 722). It, has, however been applied to the proteins themselves (Henriques and Gjalbäk, *Z. physiol. Chem.*, 1911, **71**, 511).

Biological Differentiation of Proteins

If a protein is injected into an animal, the serum of that animal acquires the property of precipitating that protein ("antigen"), forming a so-called precipitin. This precipitin is yielded only when the serum is treated with a protein derived from the animal from which the "antigen" was obtained, or from nearly allied species. The reaction is therefore characteristic of the species from which the protein was obtained rather than for the protein itself, and has been used for medico-legal investigations, and for determining the origin of meat contained in various food products. It is described in detail elsewhere (see vol. 9).

A List of the Chief Animal Proteins.—The following does not profess to be an exhaustive list of all the animal proteins which have been described; nor is it feasible in this place to give an exhaustive bibliography of the subject. As however the methods for preparing these substances vary very considerably in details, according to the materials employed, it has been thought advisable to give the references to some of the principal papers bearing on the subject. It is hoped that the selected references to original work given below will be sufficient to act as a guide to any worker who requires more details of manipulative methods than could be given in the foregoing text, in which only the most general aspects of a large subject could be treated.

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Protein	Origin	Class	References
Albumin	{ Egg (ovalbumin) crystalline. Milk (lactalbumin). Serum (crystalline) }	Albumin..... Albumin..... Albumin.....	Hopkins and Pinks, <i>J. Physiol.</i> , 1898, 23 , 130. Gurler, <i>Verh. phys. med. Gesellsch. Würzburg</i> , 1894, 1 , 143, and Inagaki, <i>Ibid.</i> , 1906, 38 , 17. Fuld, <i>Biochem. Z.</i> , 1907, 4 , 488.
Albumose	Milk.....	Proteose.....	Krawkow, <i>Arch. exp. Path. Pharm.</i> , 1898, 40 , 195.
Amyloid	Pathological tissue.....	Glycoprotein (?).....	Hanssen, <i>Biochem. Z.</i> , 1908, 13 , 185.
Arbacin	Testes of sea-urchin.....	Histone.....	Matthews, <i>Z. physiol. Chem.</i> , 1897, 23 , 399.
Bence-Jones protein.....	Pathological urine.....	Globulin (?).....	Hopkins and Savory, <i>J. Physiol.</i> , 1911, 42 , 189.
Caseinogen.....	Milk.....	Phosphoprotein.....	Hammarsten, <i>Maly's Jahrbücher</i> , 1872, 118 ; 1874, 135 and 1877, 158 . Danilewsky and Radenhausen, <i>Ibid.</i> , 1880, 186 . Haw and Gies, <i>Amer. J. Physiol.</i> , 1901, 5 , 387. Mörner, <i>Skand. Arch. J. Physiol.</i> , 1880, 1 , 210.
Chondroproteins.....	{ Bone (osseomucoid). Trachea..... Tendon (tendomucoid). }	Glycoproteins (?).....	Cutter and Gies, <i>Amer. J. Physiol.</i> , 1901, 6 , 185. (See protamines.) Numerous papers on collagens from various sources by Gies and his co-workers, <i>Amer. J. Physiol.</i> , 1901, and onward.
Clupeine.....	Testes of.....	Protamine.....	Also Sadikoff, <i>Z. physiol. Chem.</i> , 1906, 48 , 130.
Collagen.....	Connective tissues.....	Scleroprotein.....	{ Osborne and Campbell, <i>J. Amer. Soc.</i> , 1900, 22 , 422. (Langstein, <i>Beit. physiol. path. Chem.</i> , 1902, 1 , 82.
Conalbumin.....	Eggs.....	Albumin.....	Witzel, <i>Z. physiol. Chem.</i> , 1900, 29 , 388.
Concholin.....	Shells of lamelli branches.....	Scleroprotein.....	Krukenberg, <i>Ber.</i> , 1884, 17 , 1843.
Cornein.....	Coral (<i>Rhipidogorgia flabellum</i>).....	Scleroprotein.....	Mörner, <i>Z. physiol. Chem.</i> , 1894, 18 , 61. (See protamines.)
Crystallins (α and β).....	Lens of eye.....	Globulins.....	Richards and Gies, <i>Amer. J. Physiol.</i> , 1902, 7 , 93.
Cyclopterin.....	Testes of cyclops.....	Protamine.....	Huiscamp, <i>Z. physiol. Chem.</i> , 1905, 46 , 273.
Elastin.....	Ligamentum nuchæ.....	Globulin.....	Hammarsten, <i>Z. physiol. Chem.</i> , 1899, 25 , 98.
Fibrinogen.....	Blood.....	Histone.....	Kossel and Kutscher, <i>Z. physiol. Chem.</i> , 1900, 31 , 188.
Fibrinoglobulin.....	Blood.....	Scleroprotein.....	Mörner, <i>Z. physiol. Chem.</i> , 1899, 28 , 471.
Gadus histone.....	Testes of <i>Gadus</i>	Globulin (?).....	Sadikoff, <i>Ibid.</i> , 1906, 48 , 130.
Gelatin.....	Closely allied to collagen.....	Globulins.....	Schulz, <i>Z. physiol. Chem.</i> , 1898, 24 , 449.
Globin.....	Chromoprotein of blood.....	Globulins.....	Halliburton, <i>J. Physiol.</i> , 1880, 13 , 808.
Globulins.....	Animal cells.....	Globulins.....	Pohl, <i>Beit. physiol. path. Chem.</i> , 1906, 7 , 381.
	Egg.....		{ Langstein, <i>Beit. physiol. path. Chem.</i> , 1902, 1 , 82. { Osborne and Campbell, <i>J. Amer. Soc.</i> , 1900, 22 , 422
	Milk.....		{ Sebelien, <i>Z. physiol. Chem.</i> , 1885, 9 , 453.
	Serum.....		{ Various fractions of Freund and Joachim, <i>Z. physiol.</i> <i>Chem.</i> , 1902, 36 , 407, and Spiro and Fuld, <i>Ibid.</i> , 1902, 31 , 407. Wiener, <i>Ibid.</i> , 1911, 74 , 29.
Gorgonin.....	Skeletal tissue— <i>Gorgonia canollisa</i> (coral). Blood.....	Scleroprotein.....	Henze, <i>Z. physiol. Chem.</i> , 1903, 38 , 60; 1907, 51 , 64.
Hæmoglobin.....		Chromoprotein.....	Zinnoffsky, <i>Z. physiol. Chem.</i> , 1885, 10 , 16.

Protein	Origin	Class	References
Histone.....	{ Bird's blood..... Thymus.....	Histone.....	Kossel, <i>Z. physiol. Chem.</i> , 1884, 8, 511.
Ichthulin.....	Certain fish eggs.....	Phosphoprotein.....	Kossel and Kutscher, <i>Z. physiol. Chem.</i> , 1900, 31, 188. Walter, <i>Z. physiol. Chem.</i> , 1891, 15, 477.
Keratins ¹	Egg shells.....	Scleroprotein.....	Levene, <i>ibid.</i> , 1901, 32, 281. Aberghalden and Strauss, <i>Z. physiol. Chem.</i> , 1906, 48, 525.
Livettin.....	Egg yolk.....		Plimmer, <i>Trans. Chem. Soc.</i> , 1908, 93, 1500.
Lutealhistone.....	Testicles of <i>lotu</i>		Ernstström, <i>Z. physiol. Chem.</i> , 1901, 32, 351.
Myosin (paramyosinogen).....	Striated muscular tissue.....		{ Halliburton, <i>J. Physiol.</i> , 1887, 8, 331.
Myogen (myosinogen).....	Striated muscular tissue.....		Paykul, <i>Z. physiol. Chem.</i> , 1887, 12, 196.
	Bile (?) ¹		von Purth, <i>Beit. physiol. path. Chem.</i> , 1901, 1, 252.
	Egg (hyalomucin).....		Mörner, <i>Z. physiol. Chem.</i> , 1894, 18, 244.
	Cystic fluids.....		Hammarsten, <i>Z. physiol. Chem.</i> , 1882, 6, 194.
	Snail (helix pomata).....		Hammarsten, <i>Z. physiol. Chem.</i> , 1901, 32, 395.
	Synovial fluid.....		Hammarsten, <i>Pflüger's Archiv</i> , 1885, 36, 373.
	Tachinae and sputum.....		von Moller, <i>Z. physiol. Chem.</i> , 1904, 43, 145.
	Urine.....		K. Müller, <i>Z. f. Biologie</i> , 1901, 42, 408.
	Blood.....		Mörner, <i>Skand. Arch. f. Physiol.</i> , 1895, 6, 332.
	Liver.....		Webermeister, <i>Beit. physiol. path. Chem.</i> , 1900, 8, 439.
	Lymphatic organs.....		Vollsmuth, <i>Z. physiol. Chem.</i> , 1903, 37, 475.
	Meat (striated muscle).....		Bakelandt, <i>Z. physiol. Chem.</i> , 1900, 32, 342.
	Mucous membrane of stomach.....		Pekelharing, <i>Z. physiol. Chem.</i> , 1896, 22, 245.
	Pancreas.....		Pekelharing, <i>Z. physiol. Chem.</i> , 1896, 22, 233.
			α-nucleoprotein:
			Umber, <i>Z. klin. Med.</i> , 1900, 49, 464.
			Gamgee and Jones, <i>Beit. physiol. path. Chem.</i> , 1904, 4, 10.
			β-nucleoprotein:
			Hammarsten, <i>Z. physiol. Chem.</i> , 1894, 19, 191, 1902, 35, 111.
	Serum.....		Bywaters, <i>Biochem. Z.</i> , 1905, 15, 322.
	Spleen.....		Levene and Mandel, <i>Z. physiol. Chem.</i> , 1906, 47, 151.
	Suprarenals.....		Jones and Whipple, <i>Amer. J. Physiol.</i> , 1902, 7, 423.
	Thymus.....		Huiscamp, <i>Z. physiol. Chem.</i> , 1901, 32, 145.
	<i>Oesophis tubicola</i> (worm).....		Schmiedeburg, <i>Reports Naples Zool. Station</i> , 1882, 3, 373.
Omphalin.....	Testicles of fish.....	Scleroprotein.....	Pieroff, <i>Z. physiol. Chem.</i> , 1899, 28, 307.
Parathione.....	Mucous membrane of intestine of pig.....		(General) Kossel, <i>Z. physiol. Chem.</i> , 1896, 22, 178.
Protamines.....	Testis of salmon.....	Protamine.....	Siegrfried, <i>J. Physiol.</i> , 1893, 28, 319.
Reticulin.....	Testis of mackerel.....	Protamine.....	(See protamines.)
Salmine.....	Silk.....	Scleroprotein.....	(See protamines.)
Silk gelatin (sericin).....	Silk.....		Ernstström, <i>Z. physiol. Chem.</i> , 1902, 34, 481.
Silk fibroin.....	Silk.....		P. Fischer and Skita, <i>Z. physiol. Chem.</i> , 1901, 33, 177.
Spongin.....	Sponges.....		Ernstström, <i>Z. physiol. Chem.</i> , 1901, 33, 177.
Sturine.....	Testes of salmon.....		Aberghalden and Strauss, <i>Z. physiol. Chem.</i> , 1906, 48, 49.
Thyreoglobulin.....	Thyroid gland.....	Protamines.....	(See protamines.)
Vitelin.....	Egg yolk.....	Phosphoprotein.....	Oswald, <i>Z. physiol. Chem.</i> , 1899, 27, 64; 1901, 32, 121. Levene and Alsberg, <i>Z. physiol. Chem.</i> , 1901, 31, 543.

¹ The numerous proteins included under the heading, such as hair, horn, tortoise shell, are all easily prepared by separating other constituents

THE DIGESTION PRODUCTS OF THE PROTEINS

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THE GENERAL CHARACTER OF THE DIGESTION PRODUCTS

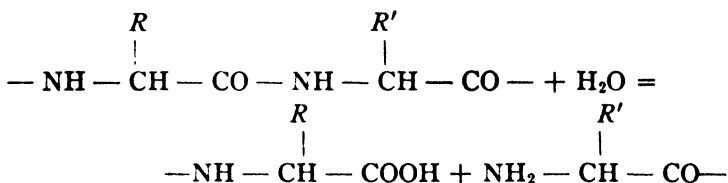
The proteins, as has been already stated (p. 621) are essentially polypeptides, which on boiling with mineral acids undergo hydrolysis, yielding as the ultimate products the amino-acids. If they are treated, however, by less drastic methods as, for example, with cold or only slightly warmed mineral acids, or with certain enzymes, hydrolysis will take place without the production of the ultimate products; in such cases substances still containing the peptide link will be produced, which themselves, on treatment by more drastic methods will ultimately yield the simple amino-acids. In some respects the degradation of the proteins is analogous to that of the polysaccharides, such as starch which by partial hydrolysis yields first the less complex polysaccharides, such as the dextrins, which themselves can be gradually degraded until only the ultimate hydrolysis products, the monosaccharides, are obtained. In one respect, however, the digestion process in the case of the proteins is far more complex than that in the case of the polysaccharides, for whereas the latter yield as ultimate hydrolysis products sometimes one and at other times two or three monosaccharides, the former give rise to a large number of amino-acids after complete hydrolytic scission. As a consequence, the degradation products of the proteins are far more complex than those of the starches and other polysaccharides.

As already stated, the intermediary degradation products of the proteins can be obtained by hydrolysis with acids at lower (room or incubator) temperatures or more usually, by treatment with certain (proteoclastic) enzymes. These latter substances, which act catalytically, are widely distributed in the animal and plant world, and

those derived from the alimentary tract of animals have been most widely studied. By the mucous membrane of the stomach of mammals is excreted pepsin, by the pancreas, trypsin, while more recently from the mucous membrane of the small intestine a third enzyme known as erepsin has been extracted. All these enzymes play an important part in the digestion of food. Furthermore, proteoclastic enzymes have been isolated from almost all organs of the body, as well as from pathological growths, and during the last few years have formed the subject of exhaustive studies (see numerous papers by Abderhalden and his pupils in the *Z. physiol. Chem.*).

As an example of a proteoclastic enzyme from plants may be cited the so-called papayotin from the papaw tree (*Carica papaya*), preparations of which are obtainable commercially (papain). Many other plants contain proteoclastic ferments.

None of these enzymes has so far been obtained in a chemically pure condition, and they act, as already mentioned, catalytically, a small quantity of enzyme being capable of hydrolysing relatively large amounts of proteins (or partially hydrolysed proteins). Their essential chemical action is to accelerate the hydrolytic scission of a peptide linkage according to the equation



Although the enzymes have not been isolated as chemical entities, they can be readily distinguished from one another by various criteria of action. Thus, for example, some proteoclastic enzymes, such as pepsin, act only in an acid medium, whereas others, such as trypsin, (pancreatin) act best in an alkaline or neutral medium. The conditions as regards acidity or alkalinity vary with the individual enzymes, each having its own characteristic condition for the exertion of its optimal activity. The enzymes, furthermore, are extremely specific with regard to their action; they will promote the peptide scission only when the hydrolysis takes place between certain definite groups. The synthesis of the polypeptides has given considerable stimulus to the study of this important aspect of diges-

tion. The action of a ferment on a peptide depends upon several factors of which the chief are the following: (a) *The nature of the amino-acid*; certain groups, such as tyrosine, are specially susceptible to the action of trypsin; and using synthetic peptides, it has been shown by Waldschmidt-Leitz and Schlatter (*Ber.*, 1927, **60 B**, 1906) that in the glycyl-glycine series, the lengthening of the chain does not affect the activity of erepsin on the peptide, whilst the introduction of a different amino-acid residue (*e. g.* leucine) into the chain renders the erepsin inactive, although trypsin is still active. (b) *The stereo-chemical configuration of the molecule*. (c) *The general configuration of the whole molecule*. As an example of this specificity may be cited the action of the pancreatic juice of the dog on various synthetic peptides (Fischer and Abderhalden, *Z. physiol. Chem.*, 1907, **51**, 264). Thus, *d*-alanyl-*d*-alanine ($d - [(CH_3)CH(NH_2)CO] - d[NH \cdot CH \cdot (CH_3)COOH]$) and *l*-leucyl-*l*-leucine undergo scission when treated with pancreatic juice, yielding *d*-alanine and *l*-leucine respectively, whereas the optical isomers, *d*-alanyl-*l*-alanine ($d [-(CH_3) \cdot (CH) \cdot (NH_2)CO] - l[NH \cdot CH \cdot (CH_3)COOH]$) and *l*-leucyl-*d*-leucine remain unacted upon. The peptide grouping appears to be capable of undergoing hydrolytic scission on treatment with enzymes, only when it is formed from such amino-acids as exist in nature. If a racemic peptide which can be digested by an enzyme is submitted to such digestion, it will undergo asymmetric scission; only those amino-acids which occur in nature are set free. Thus if the racemic alanyl leucines are digested with pancreatic juice, only the one containing the combination (*d*-alanyl-*l*-leucine + *l*-alanyl-*d*-leucine) undergoes hydrolysis, whereas the other (*d*-alanyl-*d*-leucine + *l*-alanyl-*l*-leucine) remains unchanged.

From these facts, it seems extremely unlikely that a complete hydrolytic scission of all the peptide groups contained in so complex a substance as a protein should take place, and as the individuals are formed by the conjugation of varying numbers of the numerous amino-acids, the amount of hydrolysis produced by a given enzyme will vary very considerably with the different proteins. (Cf., London and Solowjew, *Z. physiol. Chem.*, 1911, **74**, 309.) Furthermore, the enzymes differ among themselves in their predilection for attacking the peptide linkages formed by different amino-acids.

From the foregoing remarks it is obvious that the study of digestion of proteins by enzymes is an extremely complex one, and that

in actual practice the examination of a digestion mixture entails the investigation of material containing a large number of different substances.

The Preparation of Pure Digestion Products.—Although a protein digest contains, as a rule, many substances, various attempts have been made to isolate individual polypeptides in a chemically pure state.

Systematic investigations in this direction have been undertaken by Siegfried and his pupils, who employed as a precipitating reagent ferric ammonium sulphate dissolved in concentrated ammonium sulphate solution. Ammonium sulphate solution by itself precipitates the more complex products, and another series, less complex, is precipitated by Siegfried's reagent from the filtrate. By this means several amorphous substances appearing to have a constant composition and specific rotation have been obtained. In addition to these substances, the action of 12% hydrochloric acid at incubator temperature produces a series of compounds, of markedly basic character, known as *kyrines* (*Z. physiol. Chem.*, 1904, 43, 47; 1906, 48, 54; 1906, 50, 163; 1908, 58, 215).

The question of the isolation of the polypeptides from among the products of partial hydrolysis of proteins has been prominent in recent years as a result of the attempts of Abderhalden and his school to prove that the protein molecule is based on the diketopiperazine ring (see p. 622). Large numbers of peptides have been isolated in the course of this work, and some brief reference may be made to the methods adopted. The protein is hydrolysed by 70% sulphuric acid at a low temperature (*e. g.*, 20°–37°) for some days, after which the acid is removed as barium sulphate, and the filtrate is evaporated to a syrup. This is mixed with sand to form a thick paste, and the mixture is extracted in a Soxhlet apparatus with the following solvents, in the order given:—ether, ethyl acetate, acetone, methyl alcohol, chloroform and butyl alcohol. The various fractions are then evaporated and the residues, which are usually crystalline, are further separated by means of other solvents. The method is quite empirical, no rational scheme for the separation of the peptides having been developed (see Abderhalden, *Z. physiol. Chem.*, 1923, 131, 284, and subsequent volumes). A few peptides have also been isolated by other workers, by hydrolytic degradation of proteins, and many have been prepared synthetically. Some of the

products obtained from proteins are mentioned in the appended list, with the names of their discoverers. The preparation of the synthetic peptides by Emil Fischer and his pupils has resulted in a considerable to our knowledge not only of the chemical structure of the proteins, but also of the processes involved in their digestion by enzymes (E. Fischer, *Untersuchungen über Amino-säuren, Polypeptide und Proteine*, Berlin, 1906).

Polypeptides Obtained by Hydrolysis of Proteins.—

Glycyl-*d*-alanine anhydride from silk fibroin—Fischer and Abderhalden, (*Ber.*, 1906, **39**, 752)

Glycyl-*l*-tyrosine anhydride from silk fibroin—*ibid.*

	{	(Levene and Wallace (<i>Z. physiol. Chem.</i> , 1906, 47 , 143)
Glycyl-proline anhydride from gelatin		(Levene and Beatty, (<i>Ber.</i> , 1906, 39 , 2060)

<i>d</i> -Alanyl- <i>l</i> -leucine	{	from elastin—Fischer and
Glycyl- <i>l</i> -leucine anhydride		Abderhalden, <i>Ber.</i> , 1906, 39 ,
Anhydride yielding on hydrolysis alanine and proline		2315; 1907, 40 , 3544.

l-Leucyl-*d*-glutamic acid from gliadin.—Fischer and Abderhalden, (*Ber.*, 1907, **40**, 3544)

Dipeptide (phenylalanine and proline)
from gliadin—Osborne and Clapp, (*Amer. J. physiol.*, 1907, **18**, 219)

Glycyl- <i>l</i> -tyrosine	}	from silk Abderhalden, (<i>Z. physiol. Chem.</i> , 1909, 63 , 401; 1910, 65 , 417)
<i>d</i> -Alanyl-glycine		
		<i>d</i> -Alanyl-glycyl- <i>l</i> -tyrosine from silk Abderhalden (<i>Z. physiol. Chem.</i> , 1911, 72 , 1)

*iso*Leucyl-valine anhydride from casein—(Dakin, *Biochem. J.*, 1918, **12**, 290)

Hydroxyprolyl-proline anhydride from gelatin—(Dakin, *J. Biol. Chem.*, 1920, **44**, 499)

l-Leucyl-*d*-valine anhydride from casein. Abderhalden (*Z. physiol. Chem.*, 1923, **128**, 119)

l-phenylalanyl-*d*-alanine anhydride from casein—Abderhalden (*loc. cit.*)

Prolyl-leucine anhydride from gliadin—Abderhalden (*loc. cit.*)

Prolyl-glycine anhydride from gliadin—Abderhalden (*loc. cit.*)

Leucyl-serine from keratin—Abderhalden and Komm (*Z. physiol. Chem.*, 1924, **132**, 1)

L-Prolyl-d-valine anhydride—Abderhalden and Komm (*loc. cit.*)

Some of the synthetic peptides are precipitable from solution by ammonium sulphate, a property which is possessed by some of the digestion products of proteins (albumoses), which are discussed in detail below.

The Nomenclature of the Digestion Products and the Methods of Characterisation.—As, in practice, the isolation of individual digestion products and their quantitative estimation is quite unfeasible, other methods must be employed for the chemical examination and for the determination of the degree of hydrolysis and character of the hydrolysis products contained in any given mixture of digestion products.

When a protein is submitted to digestion, certain progressive changes occur in the solution. Many of the ordinary protein reactions remain unchanged, as might be expected from the fact that these are due to individual amino-acids from which the protein is built up (see p. 623). This remark refers especially to the various colour reactions. One of these, however, is especially characteristic of certain peptide groupings, and is not yielded by the simple amino-acids, viz., the biuret reaction. The colour obtained in the biuret test is somewhat different with the digestion products from that given with the proteins from which these products are obtained, having a pinker tinge. It is possible by means of enzymes to digest a protein so far that the products no longer yield a biuret reaction, although certain peptides still exist in the mixture. The biuret test is the only colour reaction which is employed for the characterisation of a digest; a biuret-free product has formed the subject of many researches on animal metabolism, and may be regarded as a characteristic product of enzymatic digestion. It can be produced by a very prolonged action of pancreatin (trypsin), or by treatment of a protein first with pancreatin and afterwards with erepsin. It contains only relatively simple products. Frankel (*J. Biol. Chem.*, 1916, **26**, 31) shows that, using 13 different proteins, pepsin-hydrochloric acid liberates 20% of the total nitrogen as amino-nitrogen in 100 hours, trypsin liberates 50% of the total nitrogen, trypsin

following pepsin liberates 70%, and erepsin following trypsin and pepsin liberates 85-90%.

Another series of changes occurs progressively during digestion, which are far more generally characteristic of the process than any of the changes in colour reactions, viz., changes in the precipitability of the digestion mixture with salt solutions.

The subject of the precipitability of proteins has already been dealt with in a former article, and attention has there been drawn to the fact that certain salts precipitate various proteins, the limits of concentration between which the precipitation occurs being characteristic both of the protein and the salt. As digestion proceeds, the limits alter, the simpler degradation products being precipitable only by higher concentrations of salts, or even remaining unprecipitated on complete saturation of the solution.

When a protein is submitted to peptic digestion in the presence of acids, an insoluble but digestible protein, such as fibrin, first becomes soluble. If, after a short period of digestion, the mixture is neutralised, a precipitate is formed which redissolves in excess of alkali, or in acid; this is the so-called "acid albumin." It may be produced by the action of acid (0.4% HCl) alone, without pepsin, at 37°, for 24 hours; a similar product, known as alkali albumin, is produced by the action of 0.1% sodium hydroxide at 37° for 18 hours, or more rapidly at 60°. The acid and alkali albumins are classed together as the *metaproteins*.

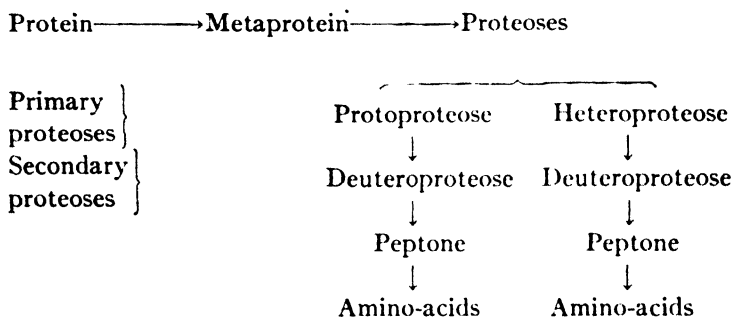
Further digestion with pepsin causes the degradation of the metaproteins, other products being formed, which are not precipitated on neutralisation of the solution. These products are, however, precipitable by salts; some are brought down by saturation with sodium chloride, others only by saturation with ammonium sulphate. Of these, the former are the more complex, and diminish in quantity as the digestion proceeds; they are known as the *primary proteoses*. The latter class, known as *secondary proteoses*, increase during the early stages of the digestion, but diminish towards the end, passing into products which are not precipitable by salts, but which still give the biuret reaction—a class of substances known as *peptones*.

The primary and secondary proteoses are not individual chemical substances, nor are the peptones. The primary proteoses are themselves divided into at least three fractions, viz. the α - and

β -protoproteoses which are easily soluble in water, and *heteroproteose*, which is less soluble in water, and is brought down by 32% alcohol. Again, three substances have been described as occurring in the fraction designated secondary proteoses, differing in their behavior towards ammonium sulphate.

The peptones are probably mixtures of the more complex peptides; they are further degraded into the simple amino-acids by the enzymes trypsin and erepsin, acting in a suitable medium, but not by pepsin.

A scheme to express the course of the degradations outlined above may be given thus:



It should be noted, however, that the actual course of digestion does not follow so regular a scheme; some of the simpler products (peptides or even amino-acids) may make their appearance quite early in the digestion, at the same time as complex bodies such as the proteoses. The above products are separated by means of salt precipitation, but the method is empirical, the amounts of individual fractions varying according to conditions.

Methods of Separation of the Digestion Fractions

(1) *Metaproteins*.—These substances are most easily prepared by the action of 0.4% hydrochloric acid at 37° for 24 hours on a solution of egg white in 10 times its weight of the acid. On careful neutralisation, the acid albumin is precipitated, but is soluble in excess of either acid or alkali. The metaprotein is also precipitable by saturation of the solution with sodium chloride or by half saturation with ammonium sulphate. Alkali-albumin is not precipi-

tated by saturation with sodium chloride. The metaproteins do not coagulate on boiling; they give the colour reactions of the proteins, as well as some of the precipitation reactions.

(2) **Proteoses:** (a) *Primary Proteoses*.—A precipitate of the primary proteoses is produced from the mixture obtained by treatment of a protein with 20 times its weight of 0.4% hydrochloric acid and a little pepsin for several days at 37°, by half saturation with ammonium sulphate. The heteroproteose is precipitated from the mixed proteoses, after redissolving in water, by the addition of alcohol up to 32%. The primary proteoses are easily soluble in water, and give most of the colour reactions of the proteins, as well as some of the precipitation reactions.

(b) **Secondary Proteoses**.—These are precipitated from the digestion mixture referred to above, after removal of the primary proteoses, by complete saturation with ammonium sulphate. They resemble the primary proteoses in most respects.

(3) **Peptones**.—The peptones remain in solution after removal of the proteoses from the digestion mixture. They are precipitated by phosphotungstic acid, tannic acid, and lead acetate, and give a characteristic pink biuret reaction. The mixture is doubtless very complex, consisting of a number of polypeptides of different composition.

Several commercial "peptones" are on the market. *Witte's peptone* is prepared by the action of pepsin on fibrin, and consists of a mixture of proteoses and peptides. Pick has separated this into the following fractions:—

Fraction	Precipitation limits with ammonium sulphate	Solubility in alcohol
Aa	24-42%	Insoluble in 32% alc.
Ab	24-42%	Soluble in 80% alcohol
Ba	54-62%	Insol. in 60-70% alc.
Bb	54-62%	Soluble in 80% alc.
Ca	70-95%	Insol. in 35% alcohol
Cb	70-95%	Insol. in 60-70% alc. (and other smaller fractions pptd between 54 and 62% saturation)
D	100% + acid	Soluble in 70-80% alc.

Based upon the above observations, one may employ the salt precipitation method for the examination of the digestion products of the proteins. As quite simple substances seem to be produced at early stages of digestion, it is perhaps somewhat illogical to divide the products rigidly into substances classed as primary proteoses, peptones, etc., as in this case the assumption seems to be made that a protein gradually undergoes degradation through all these stages. As, furthermore, each fraction obtained by salt precipitation is a complex mixture of several chemical individuals, it seems advisable to employ the precipitation process in a purely empirical manner when applying it to the examination of a digestion mixture, and to relinquish any special designation for the fractions obtained, and to indicate their origin by simply stating the degree of salt saturation at which they were precipitated. All digestion products which are precipitable by salts are designated *proteoses*; those not so precipitable are called *peptones*. In addition, the term *polypeptide* is sometimes employed. No hard and fast distinction between peptones and polypeptides can be drawn. It is perhaps convenient to confine the latter term to products which have been obtained synthetically, or which, having been obtained by a digestion process, have a known constitution. The peptones will then include these substances of polypeptide structure, which are not precipitable from salt solutions, and of which the actual chemical constitution is unknown.

In carrying out the examination of a protein digestion mixture, the following fractions may be conveniently separated:

- I. Acid or alkali albumins.
- II. Proteoses—Fraction (a) precipitated by half saturation,
Fraction (b) precipitated between 50 and 70% saturation,
Fraction (c) precipitated between 70 and 85% saturation,
Fraction (d) precipitated between 85% and complete saturation by ammonium sulphate.
- III. Peptones and amino-acids, which may be subdivided into
Fraction (a) precipitated by phosphotungstic acid,
Fraction (b) not precipitated by phosphotungstic acid.

Fraction III(a) will contain most of the peptones and the diamino-acids (see p. 638). It must be remembered that the above separation is an empirical one, and the amounts obtained in each fraction will vary slightly under different conditions, of which the most important is the concentration of the solutions precipitated. Fur-

thermore, as stated above, the limits between which the various fractions are obtained will vary with the different proteins, and it may be occasionally advisable to vary them slightly in individual cases. Nevertheless, the above scheme is a convenient one for the majority of cases; suggestions as to a standard method for carrying out the separation are given in the section dealing with experimental details. The amount obtained in each fraction is most conveniently expressed in terms of the nitrogen in each precipitate calculated as the percentage of the total nitrogen contained in the digestion mixture. The fractions obtained by salt precipitation may also be further subdivided by fractional extraction with alcohol or other solvents.

In addition to the fractionation by salts, two other quite distinct, chemical methods are employed for the examination of digestion products and estimating the degree of hydrolytic dissociation of the protein, both of which are nearly allied to one another. If a polypeptide undergoes hydrolytic scission, the number of reactive amino and carboxylic acid groups increases. This is evident from the accompanying equation, which represents the hydrolysis of the simplest peptide glycyl glycine



Whereas the polypeptide itself contains only one reactive amino and carboxylic group in the molecule, the hydrolysis products obtained from the same contain twice as many such groups as the intact polypeptide. In other words, only half the nitrogen in the unchanged polypeptide is present in an amino-group, whereas all the nitrogen in the hydrolysate is present in this form. In the case of a tripeptide formed from monoamino-acids one-third of the nitrogen is present in an amino-group, and in the products of complete scission the whole. If the scission is not complete; if, for example, only one amino-group has been split off, then the amount of nitrogen contained in the amino-groups will lie between one-third and the whole of the total nitrogen. In the scission products of a tetrapeptide, the amino-nitrogen will lie between one-fourth and the whole of the total, according to the degree of hydrolysis to which the peptide has been submitted. In general, in an *n*-peptide formed from monoamino-acids, the amino-nitrogen in the hydrolysis products will lie between $1/n$ th and the total nitrogen. Like the reactive

amino-groups, the reactive carboxylic groups will progressively increase as a polypeptide undergoes hydrolytic scission.

The percentage of nitrogen present in reactive amino groups present in a digest can be estimated by treating a known quantity of such a mixture with nitrous acid and measuring the amount of nitrogen evolved (see p. 719).

Still simpler is the method employed for following the course of digestion by estimating the changes in the number of reacting carboxylic acid groups with progressing digestion. An amino-acid, owing to its amphoteric character cannot be directly titrated by a standard alkali solution in the presence of an indicator. If, however, formaldehyde in excess be added before the titration, the amino-groups are converted into methyleneimino groups, and the substances lose their amphoteric character and can be directly titrated with standard alkaline solutions in the presence of phenolphthalein. To Sørensen (*Biochem. Z.*, 1908, 7, 45) is due this formaldehyde titration method for following the course of digestion of a protein (see also page 722). The result of the titration is usually expressed as the percentage amount of the total nitrogen in a mixture which enters into reaction with formaldehyde. Sufficient acid or alkali is first added to the mixture to render it neutral to a given indicator (*e. g.* litmus). Excess of neutral formaldehyde is then added, and owing to the reaction with the amino-groups the mixture becomes strongly acid. The additional amount of alkali necessary to neutralise this increased acidity gives the measure of the amino-groups which have entered into the reaction, which is equivalent to the number of carboxylic acid groups neutralised by the alkali.

It has been stated already that the estimation of the reactive amino-groups can also be applied to intact proteins (p. 699). The amount of nitrogen (expressed as the percentage of the total) is, however, in these cases small. Furthermore, in applying the formaldehyde titration method to these cases there is an initial difficulty in that the protein solution will as a general rule have an amphoteric character, and cannot therefore be accurately neutralised to an indicator (except in certain exceptional cases like that of caseinogen).

As the molecular weights of these substances are great, and as the number of reactive amino-groups is small, the probabilities of error

are great when the formaldehyde titration method is employed for the purposes of estimating the amino groups and obtaining a chemical factor for characterising individual proteins. It is best used for estimating the *changes* which take place during the course of a digestion, samples of the digest being withdrawn after different intervals of digestion and then submitted to the formalin titration. Initial errors due to the difficulty of accurately neutralising before addition of formaldehyde are thereby avoided. The method can also be employed for estimating the number of peptide linkages existing in a given digest, by determining the change which is produced in the formaldehyde titration number, by completing the hydrolysis of the mixture, by a known quantity of mineral acid.

The results in the above section may be summarised in the statement that it is not possible to isolate from any given digestion mixture the chemical entities which it contains. There are, however, certain analytical processes which can be applied to the examination of a digest, by means of which some idea can be formed as to the character of the material under examination. The chief of these consists in the fractional precipitation of the solutions with increasing grades of concentration of certain neutral salts, and the estimation of the percentage of the nitrogen carried down in each fraction. Although this process is of empirical nature, it gives good comparative results, and throws much light on the degree of digestion to which a given sample has been submitted. In addition to these, the amount of nitrogen precipitable by phosphotungstic acid or tannic acid can be estimated.

Furthermore, the percentage of nitrogen present in amino-groups can be estimated by determining the amount of nitrogen set free on treatment with nitrous acid, or approximately also by estimating the increased degree of acidity in the solution produced by the addition of formaldehyde. The percentage of nitrogen present in the form of peptide linkages can also be estimated by hydrolysing with mineral acids, and estimating the increase thereby produced in the "formalin-titratable" nitrogen (*i. e.*, the acidity produced by masking the basic action of the amino-groups present by treatment with formaldehyde).

The Preparation of Digests.—Digests of proteins are usually prepared by treating solutions of the substances, or when the latter are insoluble, suspensions of the same in water, with a solution or

aqueous suspension of the enzyme, at a temperature of 37.5° . For this purpose, either a thermostat or biological incubator can be employed. Care must be taken as to the reaction of the medium. The conditions of optimum action have formed the subject of numerous researches (see, e. g., Sørensen, *Enzymstudien*, *Biochem. Z.*, 1909, **21**, 131). For practical purposes, pepsin acts best in a solution containing 0.3–0.4% hydrochloric acid. During the course of digestion the acidity changes owing to the formation of amino-acids etc. The proteoclastic enzyme from the pancreas¹ acts, on the other hand, best in alkaline solution (0.2–0.4% sodium carbonate) whereas erepsin acts in neutral solution. As already mentioned in the introduction, the enzymes differ from one another in respect of the peptide groupings which they attack, and they produce different amounts of degradation with the same protein. The pancreatic enzyme possesses the capacity of breaking down certain peptide linkages which remain intact after a protein has been digested with pepsin, and erepsin possesses the property of hydrolysing peptones. By treating certain proteins with, consecutively, pepsin, trypsin and erepsin (in each case under the correct conditions as regards acidity etc.) a product which no longer yields the biuret test can be obtained, although hydrolysis is not complete, as it can be shown that the number of reactive amino-groups increases if such a digest is heated with acids.

Although the ferments act catalytically, and a small amount of ferment should break down very large amounts of protein, they often, nevertheless become inactive after the digestion has proceeded for some time. This is due to the fact that in presence of acids or alkalis at 37° they are often themselves destroyed, and it is therefore necessary, when it is required to produce the maximum change in a protein by any given enzyme, to add fresh quantities of the latter from time to time, usually at intervals of a few days.

In all cases when a digest is prepared, it is necessary to guard against bacterial contamination. For this purpose, toluene or chloroform, or both these liquids, are added to the mixture, which should be kept in closed flasks in the incubator or thermostat to prevent evaporation. The proteins should, furthermore, never be digested in too concentrated solutions or suspensions (5% is usually

¹ The pure pancreatic juice possesses no proteolytic activity, as the enzyme is present as a zymogen which can be activated, among other methods by the addition of the so-called enterokinase, which is contained in an aqueous extract of the mucous membrane of the small intestine.

more than sufficiently concentrated) as the products of digestion inhibit the action of the ferments. The amount of enzyme to be added will depend upon the quality of the preparation used. (For quantitative estimation of enzymes see page 728 *et seq.*)

To obtain the digestion products in solid form, the digestion mixture should be evaporated *in vacuo* at a temperature not exceeding 40°. The concentrated residue is then thrown into several times the volume of a mixture of equal parts of alcohol and ether; it is then rubbed up with the same mixture to free it from water, and finally washed with ether and dried *in vacuo*. When the digestion has taken place in an acid medium, an acid should be employed which can readily be removed quantitatively; peptic digests can be conveniently carried out in solutions made acid with sulphuric acid, which can be quantitatively removed by barium hydroxide when it is required to isolate the digestion products in solid form. Similarly, pancreatic digestion can be carried out in a solution made alkaline with ammonia instead of sodium carbonate.

Methods for Examination of Digestion Products and for Tracing the Course of a Digestion

For a complete examination of a digestion product (or of a sample of a digestion mixture removed after a stated interval of incubation) the following series of factors should be determined.

I. The amounts of nitrogen precipitated by (*a*) varying concentrations of certain salts, (*b*) by certain alkaloidal precipitants.

II. The percentage of nitrogen in the form of reactive amino-groups (*a*) by the nitrous acid method (*b*) by the formaldehyde titration method. By these methods also, the percentage of nitrogen still existing in the form of peptide linkages can be estimated.

III. In following the course of digestion, certain changes in the physical characters of the digestion mixtures can be studied.

In actual practice, the two first-named factors are most usually employed; the methods involved are so simple that they may easily be used as part of the routine technique in the examination of the digestion processes. In the case of the physical characters of the digestion mixtures, the chief changes measured are those of viscosity and electrical conductivity; these methods are not widely employed in the routine examination of digestion, products, and need not be

discussed in detail here (see Bayliss "*The Nature of Enzyme Action*," London, 1920). Digestive changes have also been studied by following the change of optical rotation of a digestion mixture.

Ia. The Estimation of the Nitrogen Precipitated with Varying Concentrations of Salts. (*Fractional Salt Precipitation*).—The salt most convenient for use for separating the various fractions of the proteoses is zinc sulphate; this salt can be used more advantageously than the corresponding ammonium salt, in that the filtrates from the precipitates can be directly used for the estimation of nitrogen. The reagent is, according to Baumann and Bömer, who introduced it for the purpose of proteose separations (*Z. Untersuch. Nahr. Genussm.*, 1898, 1, 106) employed in acid solution. Both to the solution of the digestion mixture and to the saturated zinc sulphate solution should be added sulphuric acid solution prepared by diluting one volume of the concentrated with 4 volumes of water. 2 c.c. of this mixture are added to each 100 c.c. of saturated zinc sulphate solution, and to each 100 c.c. of the solution to the digestion product.

The method of fractional precipitation by salts has been critically examined by Haslam (*J. Physiol.*, 1905, 32, 267 and 1907, 36, 164), who has pointed out that two sources of error are *a priori* conceivable in effecting complete separation, viz., (a) any fraction produced by a particular degree of saturation is not absolutely insoluble in a solution of the strength in which it is precipitated, and (b) a precipitate may carry down with it a certain quantity of substance which belongs to a subsequent fraction. Haslam gives methods for separating fractions of constant composition. These, however, are inapplicable to a quantitative examination of digestion products. Wiener, in investigating the separation of globulin from albumin in blood-serum by fractional precipitation with ammonium sulphate, has shown that the separation is more complete in dilute than in concentrated solutions.

The variations of the results obtained by fractional salt precipitation under different conditions of experiment, emphasize a point to which attention has already been drawn, viz., that the quantitative separation of a digest into fractions by means of salt precipitation, must be regarded as an empirical process and that it is inadvisable to regard the various fractions, to which such names as protalbumose, deuteroalbumose A, deuteroalbumose B, etc. have been assigned, as definite chemical products. Fractional precipitation,

however, if carried out under definite standard conditions, affords valuable information as to the character of the material to be examined.

The following has been found to be a convenient method for the quantitative examination of a digest by means of salt precipitation.

If the substance is a solid, it is first dissolved in water, so as to make a solution of about 10%, which is filtered clear from any undissolved matter, which is washed on a filter. The undissolved residue may be unchanged protein, and its quantity may be estimated, and the percentage of nitrogen which it contains. The clear solution is then carefully neutralised. At this point, acid or alkali albumin may be precipitated. Sometimes nucleoproteins may be precipitated at this stage, and in this case it is advisable to estimate not only the nitrogen, but also the organic phosphorus in the precipitate. The precipitate should, in any case, be washed and dried (the wash-water being added to the main bulk of the solution) and the nitrogen therein estimated, either in the whole or in a part, according to the amount precipitated. If coagulable proteins are present in the filtrate, this should be acidified with butyric acid and heated, and the coagulated protein filtered off, washed, dried and weighed, and the nitrogen contained in it estimated. If the quantity is small, the precipitate and filter paper (which should be nitrogen-free) can be directly transferred while moist to the Kjeldahl flask, and the nitrogen directly estimated without previous drying and weighing. As a rule, the quantity is only small. The filtrate, after the coagulable protein has separated, is then carefully neutralised again and nitrogen percentage estimated therein by Kjeldahl's method by making it up to a definite bulk and incinerating a small aliquot part. If the solution is too dilute, it should be evaporated. A convenient solution at this stage is one in which 10 c.c. of the solution require after Kjeldahl treatment, about 100 c.c. of $N/10$ acid to neutralise the ammonia (equivalent to about an 8.8% solution of protein). If the solution is much more dilute, it should be concentrated to about this strength. To the solution thus prepared, 2 c.c. of diluted sulphuric acid (1 volume concentrated acid, 4 volumes water) should be added to each 100 c.c. In this mixture the nitrogen should be again accurately estimated.

Into a series of 4 stoppered flasks of 100 c.c. capacity are then introduced four portions of the above solution, accurately measured

or weighed, each portion being exactly equivalent to 100 c.c. of $N/10$ sulphuric acid after treatment by Kjeldahl method. The quantity should be about 10 c.c. This solution is then diluted with varying amounts of acidified water or acidified saturated zinc sulphate solution (always 2 c.c. of diluted acid to 100 c.c. of water or salt solution). To the first flask is added 50 c.c. of zinc sulphate solution, to the second 70 c.c., to the third 85 c.c. To the fourth is added enough solid zinc sulphate to saturate the solution, which is afterwards diluted to the 100 c.c. mark with acidified saturated zinc sulphate solution. The other flasks are then made up to the mark with acidified water. They are then stoppered, thoroughly shaken, and allowed to stand. After a day, the precipitate is filtered off, and the nitrogen is directly estimated by Kjeldahl's method in 50 c.c. of the filtrates. In this way the amounts precipitated by 50, 70, 85 and 100% of saturation by zinc sulphate can be found. As 50 c.c. of the unprecipitated solution are equivalent to 50 c.c. of $N/10$ sulphuric acid, the calculations are very simple. The volume of the precipitate may be neglected. It is often inconvenient to incinerate the filtrates with sulphuric acid directly, owing to the zinc sulphate they contain. Violent bumping often occurs, and the flasks have a tendency to break. This inconvenience can be obviated by a method suggested by Salkowski (*Biochem. Z.*, 1911, 32, 355). Hydrogen sulphide is passed into the solution, and sodium carbonate solution is added from time to time to neutralise the acidity. The greater part of the zinc can thus be precipitated as sulphide, from which the organic matter can readily be removed by washing. This is not the case if the zinc is precipitated as carbonate. If necessary, the filtrate from the zinc sulphide can be concentrated before incineration. By the above method the following factors can be determined: (a) Insoluble matter (unchanged or coagulated protein), (b) acid or alkali albumin, (c) coagulable protein (d) proteoses in four fractions. In the filtrate, amino-acids and peptones exist. Some conception as to the amount of peptones in this filtrate can be formed by estimating the percentage of nitrogen existing in peptide combination. This is done by estimating the increase in the nitrogen in reactive amino-form after hydrolysis, by either the nitrous acid or the formaldehyde methods described in detail below.

In one case, which in practice is not infrequent, the above method does not yield satisfactory results *i. e.*, where gelatin is present with

the digestion products. This substance cannot be separated as a coagulable protein, and it is precipitated with salts, together with the albumoses. Various methods have been suggested for separating gelatin from the digestion products, but none has been found satisfactory. It may be recalled here that certain of the proteoses are precipitated with colloids (gum mastic or ferric hydroxide) together with the proteins, so that by these methods proteins cannot be separated from their digestion products.

(b) **The Estimation of Nitrogen Precipitated by Various Alkaloidal Reagents.** (i) *Precipitation by Phosphotungstic Acid.*—The technique in this case is exactly similar to that employed in estimating the "basic nitrogen" when determining the Hausmann numbers (see p. 693).

(ii) *Precipitation by Tannic Acid Solution.*—The reagent can be made in the following way: 100 grm. of tannic acid, 25 grm. of sodium acetate, 75 grm. of sodium chloride, and 50 c.c. of acetic acid are dissolved in water, and the solution is made up to 1,000 c.c. (Hedin). Sufficient of the reagent is added to the solution of the digestion product to separate completely the precipitable substances. Nitrogen can be estimated in an aliquot part of the filtrate.

The course of digestion can be conveniently followed by estimating from time to time the amount of nitrogen which is not precipitated by these reagents. This should increase with progressive degradation of the protein.

II. The Estimation of Nitrogen Existing in the Form of Reactive Amino Groups. (a) *The Nitrous Acid Method.*—When an amino group reacts with nitrous acid, nitrogen is evolved according to the equation $R.NH_2 + HNO_2 = R.OH + H_2O + N_2$, two atoms of the gas being evolved for each molecule of amino groups. The application of this reaction to digestion products has been already discussed (p. 712). The method now universally used is that due to D. D. van Slyke, whereby the estimation can be carried out in a very few minutes.

The apparatus employed is shown diagrammatically in the accompanying figure (p. 721).

It consists of a deaminising bulb, D, to which are attached (i) a cylindrical vessel, A, of about 35 c.c. capacity, having a mark at 7 c.c. and another at 30 c.c.; (ii) a 10 c.c. burette B; and (iii) a tap, T, serving to empty the deaminising bulb. The connecting glass tubing

is thick-walled, and has an internal diameter of 3 mm; the taps also have a bore of 3 mm. The connection between D and B should be of larger bore (8 mm). The bulb is connected through a three-way tap *c* with a large gas-burette F, of 150 c.c. capacity, the upper portion of which is graduated to 50 c.c. in tenths. The gas burette is also connected with a Hempel pipette of special type. The apparatus is usually arranged so that either the deaminising bulb or the gas pipette can be shaken mechanically, a small water-motor being convenient for the purpose.

In making an estimation, the tap T is closed, while *c* is opened to connect the bulb D with the waste; from the vessel A glacial acetic acid (7 c.c.) is run into D, followed by 30 c.c. of a 30% solution of sodium nitrite in water. The tap connecting A and D is left open, while *c* is closed; on shaking, the bulb D becomes full of nitrous oxide. When most of the liquid has been forced back into A, the tap *c* is opened, so as to allow the liquid to flow back into D and expel the gas (which will contain a certain amount of air). Again *c* is closed, and the bulb D shaken till it remains only about $\frac{1}{4}$ full of liquid (there is usually a mark on D to indicate the correct level of liquid). The tap at the base of A is now closed, *c* is opened so as to connect D to the burette F (which has previously been filled completely with water), and the solution to be examined is run in from B, a measured quantity being used. (Note that the water reservoir must be below the level of the water in F at this stage.) D is then shaken to promote the liberation of nitrogen, mixed with nitrous oxide, the evolved gas being collected in F. The shaking may be continued either for 3-5 minutes, or until a convenient volume of gas (50-75 c.c.) has been collected in F. It should be noted, however, that some of the basic amino-acids only yield the whole of their amino nitrogen after a considerable time, and the shaking may need to be continued for an hour or more. The gas remaining in D is then drawn over into F, and the whole of the gas in F transferred to the gas pipette; this pipette contains a strong solution of potassium permanganate in potassium hydroxide solution (50 grm. of KMnO_4 plus 25 grm. of KOH in 1,000 c.c. water). The pipette is shaken gently until the absorption of nitrous acid is complete, when the residual nitrogen is drawn back into the gas burette and the volume measured. The nitrogen should be returned to the Hempel pipette and shaken for a further 2 minutes, in order to ensure complete absorption of

nitrous acid, before the final measurement of volume is taken. The atmospheric pressure and temperature are noted, the volume of nitrogen is corrected to N.T.P., and from this figure the amount of amino-nitrogen in the sample examined can be calculated, by

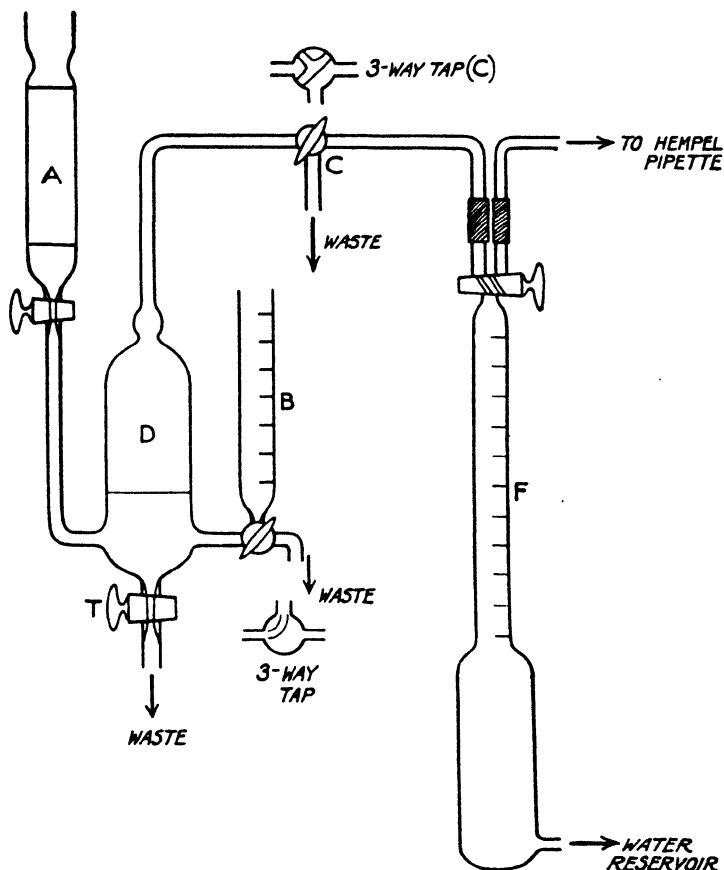


FIG. 35.—Van Slyke apparatus.

means of tables given by Van Slyke in his paper. 1 c.c. of nitrogen corresponds to roughly 0.6 mg. of amino-nitrogen; from this figure one can form an idea of the amount of liquid that should be taken for the estimation. A "blank" estimation on the reagents should be made.

In interpreting the results it must be remembered that some of the ultimate hydrolysis products of proteins do not contain all their nitrogen in the form of amino-nitrogen. Proline and hydroxyproline, for instance, give off no nitrogen on treatment with nitrous acid.

If the amino-nitrogen in a digestion mixture is estimated by this method, and an equivalent portion is hydrolysed completely and the amino-nitrogen estimated in the hydrolysed product, the difference between the two estimations is a measure of the amount of nitrogen present in the form of peptide linkages.

(b) *The Formaldehyde Titration Method.*—The theory of this method has been given already in detail (p. 712). In practice it is carried out in the following manner. To 10 c.c. of commercial formalin solution is added 1 c.c. of a 0.5% phenolphthalein solution in 50% alcohol. The mixture is then made faintly alkaline with either barium hydroxide or sodium hydroxide solution, the alkali being added till the mixture has a faint pink colour. The neutralised formalin is then added to 20 c.c. of the digestion mixture, and the whole is titrated with $N/10$ sodium hydroxide solution until a distinct pink colour is produced. It is usual to titrate to a strong pink colour, and not to the initial faint pink, as Sørensen has shown that the theoretical amount of alkali has not been added (when titrating amino-acid solutions) until the solution is distinctly alkaline. Where carbonates or phosphates are present, barium hydroxide should be used in place of sodium hydroxide. As already mentioned, the nitrogen of the free amino-groups can be deduced from a determination of the increase of acidity due to the reaction with formaldehyde, each c.c. of $N/10$ alkali solution being equivalent to 1.4 mg. of nitrogen existing in this form. When the method is applied directly to a single digestion mixture, there is a difficulty in obtaining an accurate result, arising from the circumstance that it is necessary to neutralise the solution before the addition of the formaldehyde. This cannot readily be done by titration in the presence of an indicator, owing to the amphoteric character of the mixture of amino-acids, peptides, proteoses etc. It is best applied, therefore, in estimating *changes*, when the error due to the initial neutralisation is obviated. Thus, for example, samples removed from a digest, after different periods of incubation, can be directly titrated after treatment with formaldehyde, without initial neutralisation. The

differences between the titration numbers of any two given samples removed at different intervals give an indication of the amount of digestion which has taken place within that interval.

The method is, however, best applied generally, when examining digestion products, by estimating the percentage of nitrogen still existing in the mixture in the form of peptide bonds. This can be done by titrating one portion by the formaldehyde process, and taking a similar portion, hydrolysing it in such a way as to break down completely all the peptide bonds, and titrating the hydrolysed product (after adding sufficient alkali to neutralise the mineral acid present) by the formaldehyde method. The difference of the titration numbers in the two samples is a measure of the nitrogen present in peptide form, each c.c. of $N/10$ alkali in this difference corresponding to 1.4 mg. of nitrogen thus combined.

In carrying out this hydrolysis, care must be taken that the hydrolytic scission is complete, some preparations being hydrolysed by simply evaporating once or twice on a water-bath, others requiring more drastic treatment. The technique of the process has, however, been elaborated by Henriques and Gjaldbak (*Z. physiol. Chem.*, 1910, **67**, 8). They find that the hydrolysis is best accomplished by hydrochloric acid, when it is present in the strength of three times normal in the digestion mixture, at a temperature (in an autoclave) of 150° . Under these conditions, hydrolysis is completed in 1.5 hours. The mixture thus obtained is often so highly coloured that an accurate titration is impossible. It has been shown, however, that the pigment can be removed by precipitating silver chloride in the acidified solution (Sørensen and Jessen-Hansen, *Biochem. Z.*, 1908, **7**, 407). The amount of peptide nitrogen may be estimated, therefore, in the following way:

The solution of the digestion mixture in convenient concentration (e. g., 10 c.c. containing 40 mg. N) is carefully neutralised to litmus, and then acidified with $\frac{1}{10}$ the volume of N -hydrochloric acid. Two equal portions (of say, 50 c.c.) are taken of this solution, which should be warmed in a current of air (free from carbon dioxide), to drive off any of this gas from solution. One portion is set aside, and to the other portion is added so much hydrochloric acid that the concentration is about three times normal, and the mixture is then heated in an autoclave to 150° for 1.5 hours. At the end of this period, it is transferred to to an evaporating basin, and some of the

hydrochloric acid is evaporated on the water-bath. The residue is then diluted with water, until it reaches the original bulk (50 c.c.). The same series of estimations are then carried out in both the non-hydrolysed and the hydrolysed solutions. These are (a) the total nitrogen, (b) the amount of chlorine, (c) the acidity after formalin treatment in the solution decolorised by precipitation of silver chloride. The total nitrogen and chlorine are estimated in 10 c.c. of the solution, the chlorine being determined volumetrically by Volhard's method. The difference in the chlorine in the hydrolysed and non-hydrolysed samples indicates the extra acidity due to the addition of hydrochloric acid; this should be subtracted from the formalin titration of the hydrolysed portion. The formalin titration is carried out in exactly the same manner in both the hydrolysed and non-hydrolysed portions. To 25 c.c. of the solution (already acid) are added 4 c.c. of approximately $N/2$ barium chloride solution (61 grm. $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$ per litre), and 20 c.c. $N/3$ silver nitrate solution, and water is then added to make up the total bulk of the mixture to 50 c.c. 4 additional drops of water are added to correct for the bulk of the silver chloride. The precipitated silver chloride is removed by filtration. A known volume of the clear filtrate is then titrated with alkali, after mixing it with half the bulk of neutralised formaldehyde, by the method already described. The difference in the titration numbers between the non-hydrolysed and the hydrolysed portions (after allowing for the added acid as determined by the chlorine estimation) gives the amount of nitrogen in peptide combination.

The liquids, even after treatment with silver chloride, are often somewhat coloured. Errors in titration due to this factor may be eliminated by matching a control of water coloured by the addition of dyes. For this purpose 0.2 grm. of tropaeolin O, tropaeolin OO, or Bismarck brown, or 0.02 grm. of methyl-violet in 1,000 c.c. of water may be used. To the pigmented water is then added phenolphthalein and sufficient sodium hydroxide solution to produce the requisite tint. This control serves as a standard tint in carrying out the titrations.

Silver chloride carries down a small amount of nitrogenous substance, and this is apt to cause an error. This can be determined by carefully washing the silver chloride precipitate with $N/5$ barium chloride solution, and estimating the nitrogen present therein.

There is also one other factor that must sometimes be taken into account in estimating the peptide nitrogen. In carrying out the hydrolysis a certain amount of nitrogen may be set free in the form of ammonia ("amide" nitrogen, see p. 694). This also reacts with formaldehyde to give hexamethylene-tetramine, and will therefore affect the titration. Its amount should be estimated by making just alkaline a portion of the mixture to be used for the formalin titration, by the addition of a solution of barium hydroxide in methyl alcohol (to avoid frothing) and distilling off the ammonia *in vacuo* (at a temperature of the water-bath not exceeding 40°) into an excess of standard acid. The nitrogen thus found should be subtracted from the peptide nitrogen. The amount is usually quite small.

(c) *The Alcohol-titration Method* (Foreman, *Biochem. J.*, 1920, 14, 451).—The formol titration method described above has certain disadvantages. Jodidi (*J. Amer. Chem. Soc.*, 1918, 40, 1031) discusses the method, and concludes that the results are reliable only when "the amino-acid molecule contains amino and carboxyl groups only," and that the presence of other basic groups, such as the imino-group, renders the method unsuitable. The point is further examined by Foreman (*loc. cit.*) who finds that practically all amino-acids, with the exception of glycine and asparagine, give slightly low titration values; he also calls attention to the fact that the end-point of the titration is unsatisfactory. He advocates the use of a method in which the amino-acids are titrated in a solution containing 87% of alcohol; in such a medium, the amino-group exercises no basic function, and the carboxyl group is therefore titratable. The following amino-acids give anomalous results: glutamic acid, aspartic acid, proline and arginine. Titration of the three first-mentioned may be made quantitative by using 85% acetone solution instead of alcohol; arginine in acetone solutions is quantitatively neutral.

The method is described by Foreman as follows:

A known weight of amino-acid, amino-acid mixture, or a suitable salt, is dissolved in air-free water, and made up to such a volume that the solution is about $N/10$ in respect of its carboxyl groups. A portion of 5 c.c. or 10 c.c. is titrated with $N/10$ sodium hydroxide, using phenolphthalein as indicator ("Stage I").

A further 10 c.c. portion is placed in a 250 c.c. flask, 100 c.c. of 97% alcohol and 3 drops of phenolphthalein are added, and the liquid is titrated to a clear pink colour with $N/10$ alcoholic potassium

hydroxide; the end-point is quite sharp, 0.05 c.c. of alkali producing the change of colour. A correction is made for the acidity of the alcohol used ("Stage II").

Using the same liquid as for "Stage II," 12.5 c.c. of aqueous formaldehyde, prepared by diluting colourless 40% formalin with two volumes of water, and neutralising the mixture in the presence of phenolphthalein, are added for each 50 c.c. of alcohol used in Stage II, and the titration is continued to the same end-point as before ("Stage III"). A mixture of neutralised formalin, and alcohol, in the same proportions as these have been used, is also titrated, and the figure obtained in Stage III is corrected accordingly. (The acidity of the alcohol is slightly increased by the formaldehyde.)

Using the above method, Stage I gives a quantitative result for one of the carboxyl groups in the dicarboxylic acids, and for the acid radical in such salts as histidine monochloride. Stage II, which includes the figure for Stage I, gives quantitative results for phenylalanine, tyrosine, cystine, histidine, asparagine, lysine and tryptophane. The monoamino-acids (glycine etc.) give titration values 3-7% low; proline gives a value 20-30% low, and the dicarboxylic acids give readings which are 45% low in respect of one of the carboxyl groups. In Stage III all the amino-acids give quantitative results.

85% acetone may be used in place of alcohol, in which case all the amino-acids give good titrations.

The method has been extended by Willstätter and Waldschmidt-Leitz (*Ber.*, 1921, **54B**, 2988) who have been able to titrate also the carboxyl groups of peptides, peptones and proteins. They state that these groups are titratable in the presence of 40% alcohol, while the carboxyl groups of the amino-acids require the much stronger alcohol used by Foreman. They further state that propyl alcohol is preferable in use to ethyl alcohol, but that methyl alcohol is of no value. Their paper (*loc. cit.*) gives details for estimating peptides in the presence of amino-acids.

In making an analysis of a digest it must be borne in mind that nitrogenous substances other than those derived from protein are often present. This is notably the case with digestion products of meat, the original material of which contains in addition to proteins numerous nitrogenous extractives. In such a mixture, by the method of analysis given above, the proteoses and peptones can be

estimated. It is not as a rule necessary to separate the amino-acids derived from the digestion from the nitrogenous extractives. There is no simple method at present for effecting this purpose.

THE PROTEOLASTIC ENZYMES

(See Also Article "ENZYMES," Page 151)

No enzyme has yet been prepared in a pure state, although many attempts to effect this purpose have been made; in fact, in the light of some recent work, it seems that the substances which we know as enzymes are actually systems, or associations, of two or more substances, rather than chemical entities, and that the separation of the individual components leads to the destruction of the enzyme system. Within the last few years, the enzymes have been obtained in the form of preparations of far greater activity than hitherto by the methods developed by Willstätter, Waldschmidt-Leitz and their collaborators (see papers in *Z. physiol. Chem.*, and *Annalen*, by these authors, 1921-1928).

Briefly, the methods of purification used by these workers consist in the selective adsorption of the enzymes by suitable materials, such as kaolin and specially prepared alumina, and the subsequent elution of the enzyme in a more concentrated form by means of a suitable solvent, such as a weak alkali (sodium phosphate). For experimental details of a typical case, see *Annalen*, 1921, 425, 55.

As the enzymes are not obtained in a pure state, it is not possible to ascribe definite chemical properties to them. They are identified and standardised by their digestive power.

Commercial preparations of pepsin, trypsin, erepsin and papain are on the market, as well as the well-known "liquor pancreaticus," which contains, in addition to proteoclastic enzymes, other enzymes which can act upon fats and carbohydrates.

Pepsin generally occurs in commerce as a yellowish-white amorphous powder, or in the form of scales. It is usually prepared from the mucous membrane of the pig's stomach, by extracting with acidified water containing glycerol, from which solution it can be precipitated by salts (sodium chloride or ammonium sulphate) and it can be purified by dialysis (to separate the salts) and precipitation from the dialysed solution by alcohol. It can also be prepared by extracting the mucous membrane with acidified water, evaporating the extract

to a syrup *in vacuo* at a temperature not exceeding 45°, and then scaling.

Pancreatin is extracted from the pancreas of the pig by water, or very dilute acid, and the enzymes are precipitated from aqueous solution by means of alcohol. The precipitate must be dried at a temperature not exceeding 40°. If very carefully prepared, such a preparation will contain no trypsin, but only trypsinogen, from which the activated enzyme can be liberated by the addition of minute quantities of the ferment enterokinase, which is obtained by extracting the mucous membrane of the small intestine with water. As a rule, the pancreatin becomes contaminated with enterokinase during the course of preparation.

Trypsin is prepared by extraction of the fresh pancreas of the pig by means of weak hydrochloric acid, such that the pH of the mixture is 4.7. Dilute alcohol, containing 1 c.c. of concentrated hydrochloric acid per litre, or glycerol, may also be used for extraction. Trypsin in solution is best preserved by maintaining the pH of the solution at 5.5.

Erepsin is prepared from fresh intestinal mucous membrane by extraction with 87% glycerol, and precipitation with dilute acetic acid. By adsorption on alumina from aqueous solution at pH 4.7, and subsequent elution with alkaline phosphate solution, a preparation free from trypsin is obtained (Waldschmidt-Leitz and Schaffner, *Z. physiol. Chem.*, 1926, 151, 31). From the pancreatic extract, erepsin may be separated from trypsin by adsorption of the latter on kaolin. An erepsin has been isolated from plant tissues (pineapple and pumpkin) (Willstätter, *Z. physiol. Chem.*, 1926, 152, 160).

Papain is prepared by precipitating the juice of the plant (*Carica papaya*) with alcohol.

NOTE.—Recently the name “trypsin” has been applied to the enzyme (or enzyme precursor) formerly known as trypsinogen; the activated enzyme, produced by the action of enterokinase, being termed “trypsin-kinase.”

Methods for Estimating the Proteoclastic Power of Enzymes

PEPSIN

As pure enzymes are not obtainable, only the *relative* digestive power of various preparations can be ascertained.

By the British pharmacopoeia it is officially required that pepsin should be capable of digesting at least 2,500 times its weight of coagulated egg albumin. In recent years, various other methods have been devised, which are in many respects more satisfactory than the official method.

(a) *Pharmacopæial Method*.—The egg albumin employed for the test is made by boiling fresh eggs for 15 minutes, cooling, separating the whites from the yolks and membrane, and after drying the former with a cloth, sieving it through a wire gauze containing 12 meshes to the cm. If 12.5 gm. of this albumin are suspended in 125 c.c. of acidified water, prepared by mixing 1 gm. of hydrochloric acid of sp. gr. 1.160 with 156 c.c. of water (0.2% HCl), and 5 mg. of pepsin are added to the mixture and the whole incubated for 6 hours, with frequent shaking, at 40.5°, the protein should dissolve, with the exception of a few flakes, to a clear solution. The relative strengths of different preparations can be approximately ascertained by estimating how much more or how much less than 5 mg. can produce the same result.

(b) *Mett's Method*.—In this method, egg-white is also used as a substrate for investigating the action of enzymes, but the method of manipulation is different. The fresh egg-white is drawn up into small tubes of about 2 mm. bore (care being taken that no air-bubbles are drawn up at the same time), which are then placed in nearly boiling water for 2 to 3 minutes; the egg albumin is thus coagulated. The tube is then divided into equal portions of about 10 mm., care being taken not to break up the coagulum when the lengths of tube are divided up from one another. Two or three lengths are then introduced into small flasks containing 0.4% hydrochloric acid solution, and a known quantity of the enzyme solution dissolved in a volume of water equal to that of the acid solution. After 8 to 10 hours, the tubes are removed, and the length of the albumin dissolved from the ends of the column is measured by a scale. When comparing two pepsin solutions experiments are carried out in two similar mixtures, and the square roots of the lengths digested are approximately proportional to the digestive activity of the enzymes.

This method has been very largely used, but in recent years, somewhat more convenient methods have been introduced, in one of which coagulated egg-white is used as a substrate (*Hata's method*), and in the other edestin (*Fuld's method*).

(c) *Hata's Method* (*Biochem. Z.*, 1910, 23, 179).—The substrate in this method is prepared in the following way. Egg-white is rubbed up in a basin until it is of a uniform consistency. Five times the volume of water is then added in small portions at a time, the mixture being well stirred after each addition. The solution of egg-white is then filtered through gauze, and heated in a water-bath at 60° for about 20 minutes. A homogeneous turbid mixture is thus obtained which can be kept under toluene. The mixture is diluted with 9 times its volume of water before use. 5 c.c. of the diluted mixture are now introduced into a series of tubes to each of which is added 1 c.c. of 0.4% hydrochloric acid; then to each tube are added varying portions (0.2, 0.4, 0.6 etc. c.c.¹) of the enzyme solutions containing known quantities of the enzyme preparation, and the tubes are incubated in a water-bath at 40° for 15 minutes. The tube is then noted in which the smallest amount of enzyme has produced clarification within this time. If, for example, 0.2 c.c. of enzyme A and 0.4 c.c. of enzyme B have been just sufficient to produce this effect, then enzyme A is twice as active as enzyme B. That is, the active strength of an enzyme solution is inversely proportional to the amount necessary to produce clarification of the substrate within 15 minutes.

Biedermann recommends the following substrate for the detection of small traces of pepsin: Dry egg-albumin is dissolved in water, a little acetic acid and sodium chloride added, and the mixture is boiled to coagulate the albumin. The flocculum is filtered, washed and pressed, and rubbed into a thick paste with glycerin, in which state it can be preserved indefinitely. A small piece of this paste, shaken with 10 c.c. of water, forms a milky fluid, not settling for several hours, and constitutes the substrate.

Fuld's Method (Fuld and Levison, *Biochem. Z.*, 1907, 6, 473, and Blum and Fuld, *ibid.*, 1907, 14, 62).—In this process edestin, the crystalline protein from hempseed, is used as the substrate. This substance, on treatment with hydrochloric acid, is converted into edestan, which is thrown out of solution on the addition of sodium chloride. If pepsin is present, the edestin is digested; if the ferment is present in sufficient quantity, then, after a given interval, the addition of sodium chloride no longer produces a precipitate. To carry out the reaction, portions of 5 c.c. of a 0.5% solution of edestin

¹ If less than 0.2 c.c. produces the full enzymic effect, the solution should be diluted. If 1 c.c. is insufficient, the solution should be made stronger.

in 0.4% hydrochloric acid are introduced into a series of test-tubes to which varying amounts of enzyme solutions (0.1, 0.2, 0.3, etc. up to 1 c.c.¹) are added. After keeping for 1 hour at ordinary temperatures, 1 c.c. of saturated sodium chloride solution is added to each tube. The tube is noted which remains clear under these conditions with the smallest amount of enzyme present. The activity of two enzyme solutions will be inversely proportional to the smallest volume of the solution which will digest the edestin solution so far that, under the specified conditions of experiment, no precipitate is produced with sodium chloride within 1 hour.

A modification of this method has been described by Ege (*Z. physiol., Chem.*, 1923, **127**, 125).

Grützner's Method.—In this method fibrin is used for the substrate. In the original process of Grützner the fibrin was stained with carmine which, as digestion proceeded, was set free and coloured the supernatant liquid. This pigment can only be used in acid media, as it is dissolved out by alkalies, and for this reason, Congo Red has been substituted, as this dye can be used in both acid and alkaline media. The principle of the method consists in determining colorimetrically the amount of dye-stuff set free when the same quantities of coloured fibrin are acted upon by the same volumes of the enzyme solutions under given conditions in a given time. Smorodincev (*Z. physiol. Chem.*, 1925, **149**, 173) uses fibrin dyed with diphenylosaniline in glycerin solution, the amount of digestion being determined by colorimetric comparison with standard solutions of the dye in glycerin. The method may be extended to the estimation of activity of any proteoclastic enzyme.

Voigt's Method (*Biochem. Z.*, 1923, **142**, 101).—In this method 1 c.c. of blood serum is mixed with 8 c.c. of *N*/10 hydrochloric acid and 1 c.c. of pepsin solution. After incubating for 24 hours, 0.3 c.c. of the mixture is diluted with water to 20 c.c., and 2.5 c.c. of sulphosalicylic acid are added. The resulting opalescence, which is due to the precipitation by the acid of undigested protein, is measured by means of a nephelometer.

TRYPSIN

The test for the tryptic activity of pancreatin suggested in the British Pharmacopoeia Codex, is the following: 28 cgrm. of the

¹ See preceding footnote.

preparation and 1.5 grm. of sodium hydrogen carbonate¹ are added to tepid water contained in a flask, and this solution is added to 400 c.c. of milk, previously warmed to 38°; the mixture is kept at this temperature for 30 minutes; the milk should in this time be so far digested that a sample, on mixing with nitric acid, yields no coagulum of protein.

Trypsin preparations can also be compared by Mett's method, as given above for pepsin. Gelatin coloured with an aniline dye (methylene blue) may be conveniently substituted for egg-white, the gelatin being drawn into the tubes while warm and allowed to set. The digestion must be carried out in an alkaline medium (0.4% sodium carbonate).

The digestive activity of preparations can also be compared by measuring the rate of digestion of a 4% solution of caseinogen in 0.4% sodium carbonate solution by Sørensen's formaldehyde titration method, samples of the digest being removed from the digestion mixture and titrated from time to time (see p. 722). The strengths of the preparations can be compared by finding the relative amounts necessary to produce a given amount of change in a certain time. This is perhaps the most convenient method for comparing tryptic activities.

Robert's Method.—30 c.c. of fresh milk are diluted with 30 c.c. of water in a small flask, and warmed to 40°; 1–5 c.c. of the trypsin solution (usually about 0.1%) are added. At the end of every 30 seconds, a portion of 3 c.c. is withdrawn and heated to boiling. It is noted at what time a curdling or precipitation occurs; the activities of two samples of trypsin are proportional to the times taken, respectively, to produce the coagulation, under the same conditions of temperature and concentration. Vernon considers the method very exact if the values are corrected by a table (*J. Physiol.*, 27, 182).

EREPSIN

According to Waldschmidt-Leitz and Schaffner (*Z. physiol. Chem.*, 1926, 151, 31) erepsin has an optimum activity at pH 7.8. It is best estimated by alkalimetric titration in 85% methyl alcoholic solution of the carboxyl groups liberated by the action of the enzyme on glycylglycine.

¹ Some commercial products contain sodium carbonate.

PAPAIN

According to the British Pharmacopœia Codex, a good sample should digest 200–250 times its weight of blood fibrin in 4–5 hours at a temperature of 45–50°. It can act in both slightly acid and alkaline media.

THE PLASTEINS OR COAGULOSSES

When ferments are allowed to act upon digestion products, in concentrated solutions, precipitates known as plasteins or coaguloses are formed, of which the nature is not yet definitely known, in spite of the fact that they have formed the subject of a large number of researches. (See Wasteney and Borsook, *J. Biol. Chem.*, 1924, **62**, 15.) The precipitate is best produced at pH 4.0; on altering the reaction of the medium to pH 1.7, the substance is again hydrolysed. The plasteins possess the coagulability and precipitability of the albumins, and apparently have molecular weights of the same order. It is suggested that their formation is due to the synthetic action of the enzyme, which is able to accelerate a building-up process in concentrated solutions of the degradation products. Gelatin hydrolysates have not been made to produce the plastein.

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